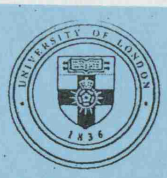

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REGIO- AND ENANTIO-SELECTIVE BIO-OXIDATIONS: *CUNNINGHAMELLA ECHINULATA* FOR BAEYER-VILLIGER.

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A thesis submitted to the University of London for the degree of Doctor of Philosophy

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I, Clive Stuart Whitcher, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

The monooxygenase catalysed Baeyer-Villiger reaction provides a method of synthesising enantiopure products which are otherwise difficult to obtain by other strategies. In this thesis the fungus *Cunninghamella echinulata* NRRL 3655 has been shown to synthesise a Baeyer-Villiger monooxygenase enzyme (CeBVMO) which is known to catalyse the insertion of an oxygen atom in to the C-C(=O) bond of a cyclic ketone, forming a lactone group. Activity was followed using racemic (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone as the test substrate. Interestingly, this CeBVMO performs regioselective oxidation of the (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone predominately producing the (-)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one lactone and to a much lesser extent (-)-(1*S*,5*R*)-2-oxabicyclo[3.30]oct-6-en-3-one in a 20:1 ratio. The same lactone enantiomers are produced when using the cyclohexanone monooxygenase, cloned and over expressed in *E. coli* from *Acinetobacter calcoaceticus* (AcCHMO), when applied to racemic (+/-) bicyclo[3.2.0]hept-2-en-6-one. However, unlike the CeBVMO this AcCHMO system performs a regiodivergent oxidation producing both lactone regioisomers in a 1:1 ratio which requires further chromatographic separation of the lactones. This separation step can be avoided when using the CeBVMO due to the difference in lactone regioselectivity making this system highly desirable.

In this thesis the growth characteristics of *C. echinulata* were studied and growth by submerged culture on a 15L scale allowed partial purification of the CeBVMO enzyme. Two further enzymes were found, an alcohol dehydrogenase and lactone hydrolase which make up part of a multi step pathway system in combination with the CeBVMO. Investigation into the substrate specificity of the CeBVMO and alcohol dehydrogenase was carried out using a range of cyclic ketones and alcohols and the use of these enzymes as part of a multi step pathway were studied using enantiomerically pure ketones as starting substrates.

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ABBREVIATIONS

Units and processes

Cat-ELISA	Chloramphenicol Acetyl Transferase Enzyme Linked Immunosorbent Assay
C.E	Capillary Assay Electrophoresis
°C	Degrees Celsius
DOT	Dissolved Oxygen Tension
ELISA	Enzyme Linked Immunosorbent Assay
FRET	Fluorescence Resonance Energy Transfer
FPLC	Fast Protein Liquid Chromatography
g	Grams
GC	Gas Chromatography
h	Hour
HPLC	High Pressure Liquid Chromatography
L	Litre
mg	Milligrams
mL	Millilitres
µm	Micometre
µL	Microlitres

µmol	Micromoles
M	Molar
mM	Millimolar
mmol	Millimoles
MS	Mass Spectrophotomerty
ng	Nanograms
nm	Nanometres
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
QUEST	Querying for enzymes using three-hybrid system
rt	Room Temperature
TLC	Thin Layer Chromatography
UV	Ultra Violet
V	Volts
vvm	Vessel Volume per Minute, aeration

Compounds, proteins and reagents

ACE	Angiotensin Converting Enzymes
AcCHMO	*Acinetobacter*-Cyclohexanone 1,2-Monooxygenase
AEBSF	(4-(2-aminiethyl)benzenesulfonyl fluoride hydrochloride)
BSA	Bovine Serum Albumin
BVMO	Baeyer-Villiger Monooxygenase
CeBVMO	*C. echinulata*-Baeyer-Villiger Monooxygenase
CHMO	Cyclohexanone 1,2-Monooxygenase

CPO	Cytochrome P450 oxidoreductase
DNA	Deoxyribonucleic Acid
E-64	(trans-epoxysuccinyl-L-leucyamido(4-guanidino)butane)
dNTPs	deoxyribonucleoside triphosphates
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide
GST	Glutathione S-Transferase
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
NADH	Nicotinamide Adenine Dinucleotide, Reduced form
NADPH	Nicotinamide Adenine Dinucleotide Phosphate, Reduced form
pNPHOAc	*para*-nitro phenyl acetate
PPG	Polypropylene Glycol
RNA	Ribonucleic Acid
SDS	Sodium Dodecylsulfate

Microbial collections

ACM	Australian Collection of Microorganisms, Department of Microbiology, University of Queensland
ATCC	American Type Culture Collection
NCIMB	National Collections of Industrial and Marine Bacteria
NRRL	Northern Regional Research Laboratory

CHAPTER 1

Introduction

1.1 Introduction

The main objective of this research was to study the Baeyer-Villiger monooxygenase present in the fungus *Cunninghamella echinulata.* This Baeyer-Villiger reaction is known to catalyse the bioconversion of bicyclic ketones containing a cyclobutanone structural motif. Currently, a cyclohexanone monooxygenase system cloned from *A. calcoaceticus* exists in a recombinant *E. coli* host displaying regiodivergence and enantioselectivity towards bicyclic ketones. In this thesis the CeBVMO found in *C. echinulata* is used as an example system that displays regioselectivity and enantioselectivity towards bicyclic ketones and in combination with two further pathway enzymes has the ability to perform multistep reactions.

In this first chapter, the main aspects of biocatalysis are addressed. The use of whole cells or isolated enzyme systems as biological catalysts that have the ability to perform beneficial chemical reactions which, by conventional chemical techniques are difficult, inefficient and undesirable for large scale processes.

1.2 Biocatalysis

1.2.1 Introduction

Over the past 50 years the use of biocatalysts to promote desirable reactions has increased rapidly within a variety of industrial processes such as the chemical manufacture of viable products, agricultural based products and the pharmaceutical industry (Straathof *et al.*, 2002). The first large scale biochemical engineering process was performed in 1947 by Merck with the production of streptomycin (Chaudhuri, 1997). Since then, the interest and expertise in biotechnology has increased. Biological catalysts are appealing as they are often efficient and selective. The specific reactions biocatalysts perform can be divided in to four categories.

Substrate selectivity

The ability to distinguish subsets of compounds within a group of chemically related compounds, e.g. the broad range transaminase isolated in *E. coli* which produces amino acids from 2-ketoacids using L-aspartic acid or L-glutamic acid as the amino group donor, driven by the conversion of oxaloacetic acid to pyruvic acid. (Crump *et al.*, 1992). This transaminase selectivity uses 2-ketoacids but does not act on pyruvate despite their structural similarities, figure 1.1.

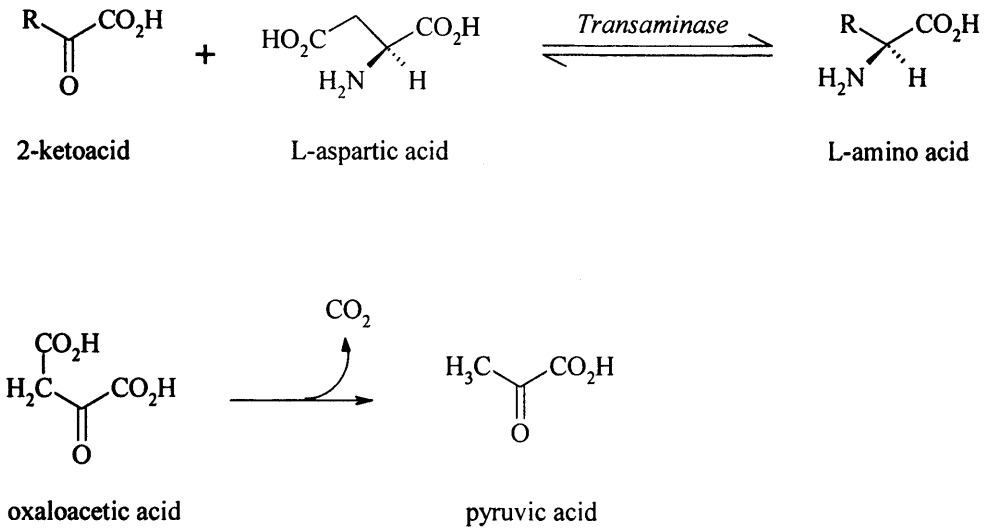
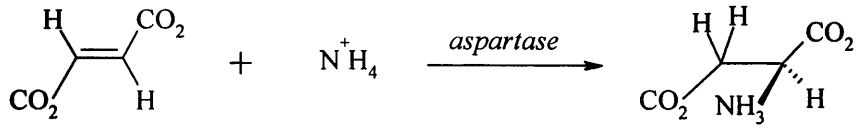


Figure 1.1: Transaminase reaction. Conversion of 2-ketoacid to L-amino acid driven by the conversion of oxaloacetic acid to pyruvic acid

Stereoselectivity

The ability to act on a single enantiomer or diastereomer selectively, e.g. resolution of racemic mixtures or stereoselective addition. For example, the production of aspartame relies on the stereoselective addition of ammonia to fumaric acid to yield L-aspartic acid, one of the key components, by the enzyme aspartase, figure 1.2 (Chibata *et al.*, 1986).

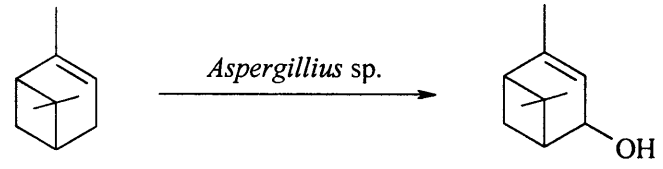


fumaric acid L-aspartic acid

Figure 1.2: Conversion of fumaric acid to L-aspartic acid by the enzyme aspartase

Regioselectivity

The ability to act on one distinct location in a molecule, e.g. hydroxylation reactions. For example, the hydroxylation of α-pinene to the mint flavour verbenol using an induced mutation strain of *Aspergillus* sp. (Agrawal *et al.*, 1999) figure 1.3.

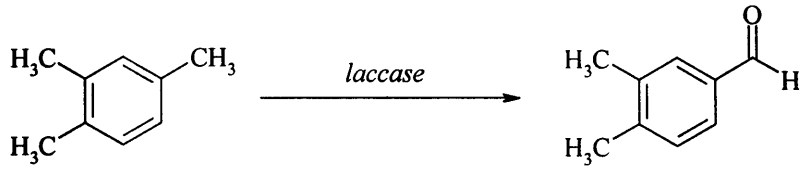


α-pinene verbenol

Figure 1.3: Hydroxylation of α-pinene to verbenol by a mutated strain of *Aspergillus* sp.

Functional group selectivity

The ability to act on one functional group in the presence of other functional groups for example the conversion of methyl aromatic compounds to aldehydes by the enzyme laccase isolated from the fungus *Trametes versicolor* (Fritz-Langhals *et a.*, 1998) figure 1.4.



1,2,4-Trimethyl-benzene 3,4-Dimethyl-benzaldehyde

Figure 1.4: Conversion of 1,2,4-trimethyl benzene in to 3,4-dimethyl benzaldehyde using the enzyme laccase isolated from *T. versicolor*

Biocatalytically, catalysts can either be isolated as free enzymes or contained within the biological cell.

Using catalysts as free enzymes allows for a simpler process since the number of side chain reactions is reduced. High rates of activity are observed, often faster than the same reaction in the absence of the biocatalyst. Enzymes operate under relatively mild conditions therefore allowing products to be generated, which under extreme conditions created by chemical synthesis are unstable.

However, enzymes do have disadvantages. They are often sensitive to substrate and product concentrations, may be unstable outside of a whole cell system and those with high energy requirements will require expensive high energy cofactors (NADH / NADPH). In order to overcome this energy requirement, catalysts contained within biological cells may be used as cofactor supply is not needed as cofactor recycling occurs within the cell.

The enzyme is often more robust and less sensitive to environmental changes. Large amounts of the enzyme may be obtained by fermentation followed with cell recovery by filtration, which is a relatively simple process. However, some of the biggest disadvantages are those of low product yield often caused by other cellular metabolic pathways, risk of contamination and purification of final products from media containing other metabolites.

Enzymes can be classified into six main groups according to the type of reaction catalysed set out by the International Union of Biochemistry (NCIBUB, 1991).

Oxidoreductases EC1

These enzymes catalyse redox reactions in which hydrogen or oxygen atoms are transferred between molecules. This extensive class includes the dehydrogenases (hydride transfer), oxidases (electron transfer to molecular oxygen), oxygenases (oxygen transfer from molecular oxygen) and peroxidases (electron transfer to peroxide). For example lactate dehydrogenase, conversion of pyruvate into lactate, figure 1.5

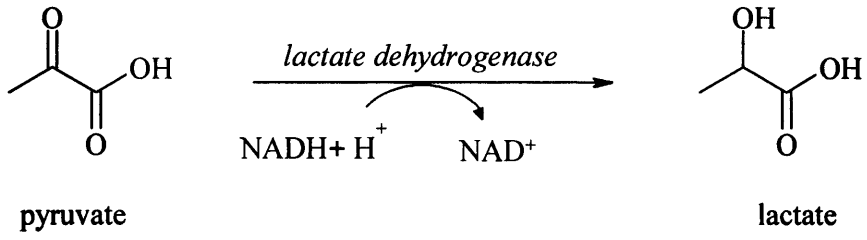


Figure 1.5: Conversion of pyruvate into lactate by the enzyme lactate dehydrogenase

Transferases EC2

These enzymes catalyse the transfer of an atom or group of atoms (e.g. acyl-, alkyl- and glycosyl-), between two molecules, but excluding such transfers as are classified in the other groups (e.g. oxidoreductases and hydrolases). For example aminotransferases and the conversion of acetophenone in to phenylethylamine, figure 1.6.

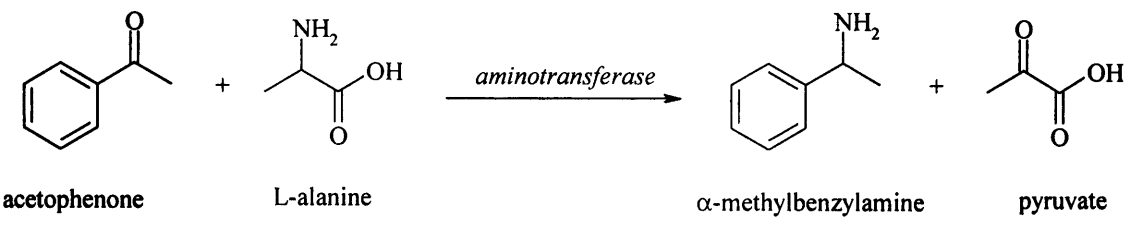


Figure 1.6: Conversion of acetophenone to α-methylbenzylamine by an aminotransferase

Hydrolases EC3

Enzymes which involve hydrolytic reactions and their reversal. This is presently the most commonly encountered class of enzymes within the field of enzyme technology and includes the esterases, glycosidases, lipases and proteases (Straathof *et al.*, 2002). For example, lipases are the most used enzymes in organic synthetic chemistry due to their regio and steroselective hydrolysis of carboxylic acid esters (Reetz, 2002). The reaction can either be lipase catalysed esterification or transesterification, figure 1.7.

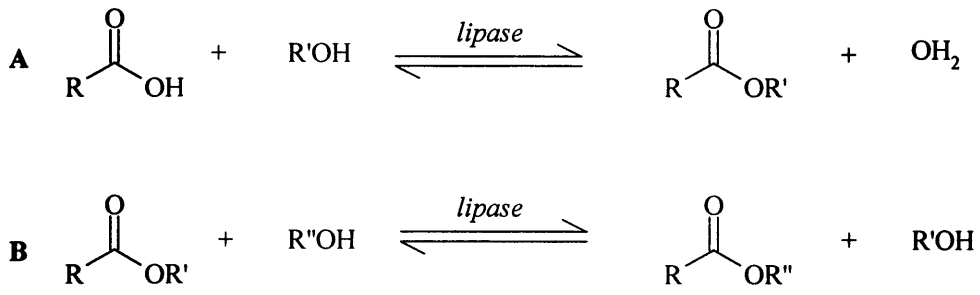


Figure 1.7: Lipase catalysed A- esterification and B- transesterification

Lyases EC4

Involve elimination reactions in which a group of atoms is removed from the substrate resulting in the formation of double bonds or the addition of chemical groups to double bonds. This includes the aldolases, decarboxylases, dehydratases and some pectinases but does not include hydrolases. One such example is that of the aspartate ammonia lyase from *Pseudomonas fluorescens* (Takagi *et al.*, 1986) figure 1.8.

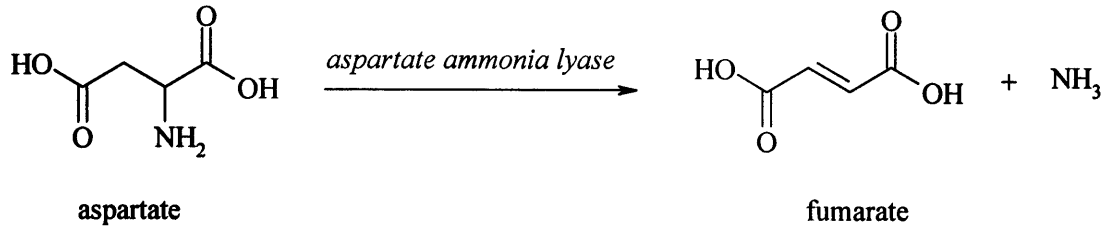
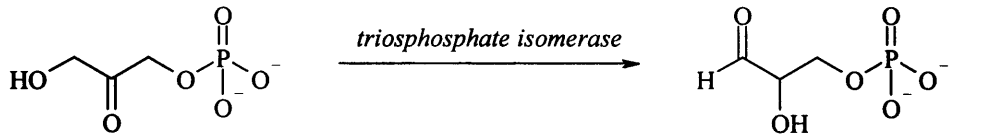


Figure 1.8: Conversion as aspartate to fumarate via a lyase catalysed reaction

Isomerases EC5

Isomerases catalyse molecular isomerisations and includes the epimerases, racemases and intramolecular transferases. For example, the triosephosphate isomerase isolated from chicken (Straus *et al.*, 1985) catalysing the conversion of dihydroxyacetone phosphate to glyceraldehyde-3-phosphate, figure 1.9.



dihydroxyacetone phosphate glyceraldehyde-3-phosphate

Figure 1.9: Conversion of dihydroxyacetone to glyceraldheyde-3-phosphate by triosephospahte isomerase

Ligases EC6

These enzymes also known as synthetases which form a relatively small group of enzymes involving the formation of a covalent bond joining two molecules together, coupled with the hydrolysis of a nucleoside triphosphate.

1.2.2 Screening for biocatalysts

The use of biocatalysts has provided an invaluable tool for the organic chemist and the creation of new organic molecules can be carried out with high yield, high efficiency and with few by-products. However, their outstanding properties are the ability to perform regioselective and stereospecific reactions and high demands from industry continue to push the boundaries to find more selective and efficient catalysts.
Biocatalysts are used in many manufacturing sectors and reports estimate sales range from $120M to 1.8 billion depending on market sector (Comyns, 2002).
As well as being used in manufacturing, biocatalysts have a lead role in reducing environmental pollution, in so called 'green chemistry'. Companies have turned to green chemistry as a way to reduce or eliminate hazardous substances generated during the design, manufacture or application of chemical products. Approaches to tackle pollution problems associated with conventional chemical synthesis have ranged from the use of benign solvents to biodegradable products (Anastas *et al.*, 2000).

To successfully exploit biotechnology one must first find a suitable biocatalyst. The catalyst must provide a viable synthetic step within the production of a product, which itself must be of use. A suitable process for the product may be already be in place so the integration of a biocatalytic step into a process must be efficient, cost effective, safe to use and free to use as regards to patents. Achieving good results is crucial to maintain profitability of the product, satisfying regulatory approval and patenting necessities.
In order to discover new biocatalysts vast libraries of microorganisms are screened for specific activity towards a substrate of interest. In order to successfully screen the libraries, instrumental methods may be employed for the detection of biocatalysts, directly monitoring the reaction of interest. Examples include HPLC, capillary array electrophoresis and ELISA based systems. Although these methods assay selective substrate binding or product binding proteins other methods to monitor the progress of a reaction are available such as chromometric and fluorimetric assays. Simple markers are linked directly to the catalytic intermediate providing a simple reliable test. An example of this is detailed by Klein and co-workers using an enantioselective fluorogenic assay for alcohol dehydrogenase (Klein *et al.*, 1999; figure 1.10).

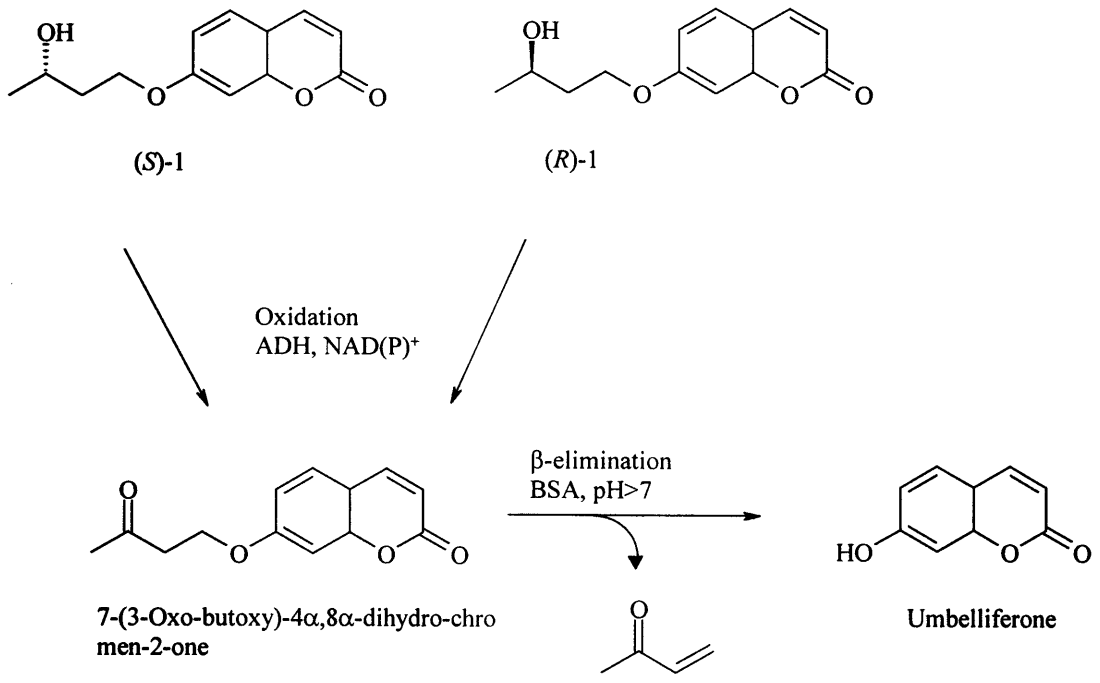


Figure 1.10: The ketone 7-(3-oxo-butoxy)-4α,8α -dihydro-chromen-2-one formed by the oxidation of either (S)-1 or (R)-1 7-(3-hydroxy-butoxy)-4α,8α -dihydro-chromen-2-one which undergoes β-elimination under catalysis by BSA to give the fluorescent product umbelliferone which has fluorescence at λ_{em}=460 nm.

A range of methods developed in recent years for high throughput screening are detailed in table 1.1 below.

Table 1.1: Methods for screening microorganisms adapted from Wahler *et al*, 2001

Method Type	Endpoint	References
Chromometric/ Fluorimetric	TLC	Reymond *et al.*, 1996
	Product staining	Taylor *et al.*, 1999
	Biosynthesis of colour products	Schmidt-Dannert *et al.*, 2000
Sophisticated reagents	Cat-ELISA	Tawfik *et al.*, 1993; Geymayer *et al.*, 1999
	Competitive cat-ELISA	Taran *et al.*, 1999
Sophisticated instruments	HPLC	Hirose *et al.*, 1993
	MS	Greenbaum *et al.*, 2000; Grüninger-Leitch *et al.*, 2000
	CE	Reetz *et al.*, 2000

1.2.3 Improving existing biocatalysts

As the popularity of biocatalysts increases it is necessary to improve existing ones or to find new ones. Current biocatalysts have limitations, for example, the availability of enzyme, stability in buffered systems and other inorganic environments such as ionic liquids and narrow substrate specificity where engineering enzymes to utilise alternative carbon sources, either those used to synthesise commercially important products or for biodegradation breaking down environmentally dangerous compounds would be highly advantageous.

The most common method for improving biocatalysts is by changing the genetic code by introducing mutations and many techniques have been developed for randomly introducing point mutations in to DNA segments. Random mutations have been introduced along a stretch of DNA using error prone polymerase chain reaction (PCR), chemical mutagenesis, UV irradiation or poisoned nucleotides. The most used technique is that of error prone PCR. This method is essentially the same as performing a conventional PCR reaction with intentional errors introduced by varying the concentrations of Mn^{2+} and dNTPs which increases the mutation rate between 2 and 8 mutations per 1000 base pairs. However, it is important that the mutation frequency is tightly controlled, as very few mutations are beneficial and therefore it is easy to swamp favourable mutations with useless ones if the error frequency is too high. The opposite also applies; Too few errors and the wild type sequence will dominate the population (Zhao, 1997).

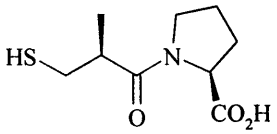
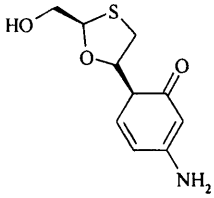
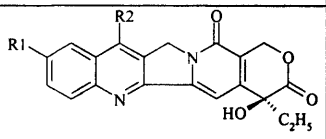
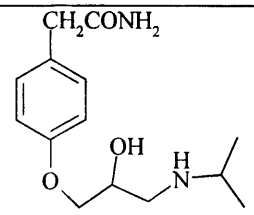
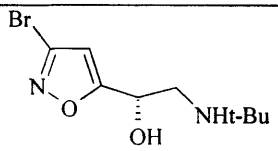
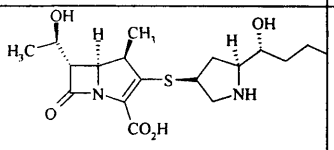
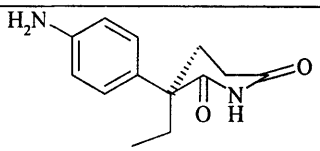
Another very useful method is that of site directed mutagenesis which is a more rational approach requiring some knowledge of the enzyme structure. This process has been lucrative for improving enzyme stability towards thermal inactivation and oxidation. Improvements in thermostability have resulted in the removal of asparagine residues in α-amylase (Declerck *et al.*, 2000) and the addition of proline in to α-amylase to provide a more rigid structure (Zhu *et al.*, 1999). Improvements with respect to the oxidation of sensitive thiol residues has resulted in the removal of cysteine and methionine residues (Slusarczyk *et al.*, 2000).

1.2.4 Applications of biocatalysts

The main explosion of interest in biocatalysis has originated from the pharmaceutical industry where the use of biological catalysts has provided the synthesis of drug metabolites and key intermediates. The pharmaceutical industry accounts for over half of the total industrial sectors that use bioconversion products (Straathof *et al.*, 2002). Drug discovery has long relied on the modification of promising starting materials from existing chemicals. The journey from the discovery of a pharmaceutical substance to its commercial production is a slow and costly process in which stringent regulatory requirements demand safety, stability and potency to be demonstrated, together with a reliable manufacturing process and a rigidly controlled production environment.

Today many drugs such as anti-cancer and anti-virals are synthesized using enzymes. One of the first optically pure synthetic drugs produced was Aldomet® by Merck (reviewed in Buckland *et al.*, 2000). Since this first success many other drugs have been synthesised effectively on a laboratory scale as well as large scale applications. Table 1.2 details some drugs that have successfully been made on a laboratory scale.

Table 1.2: Examples of optically pure drugs that have been successfully synthesised on laboratory scale using biocatalysts

Product name	Uses	Microorganism used	Structure of product	Reference
Captopril	ACE	*Pseudomonas* or *Aspergillus* lipase		Cushman *et al.*, 1977
Lamivudine	Antiviral nucleoside	*Pseudomonas* lipase		Milton *et al.*, 1995
Camposar	anticancer	*Pseudomonas* lipase		Patel *et al.*, 1992
Atenolol	β-blocker	*Pseudomonas* lipase		Bevinakatti *et al.*, 1992
(S)-Broxaterol	β_2-adrenergic stimulant	Thermostable alcohol dehydrogenase from *Thermoanaerobium brokii*		De Amici *et al.*, 1989
MK0499	New broad spectrum β-methyl-carbapen antibiotic	*Mortierella alpina*		Chartrain *et al.*,1995
Eplipten (amino-glutethimide)	antimetastatic	Bakers yeast		Fogliato *et al.*, 1995

The large scale synthesis of pharmacologically active molecules has become more viable over the past few years. Today many drugs that are in worldwide use are now manufactured from compounds produced from biocatalytic routes. Examples of these are L-DOPA and Glipizide.

L-DOPA (3,4-dihydroxy-L-phenylalanine) is an anti Parkinson drug. It is a precursor of dopamine which is naturally found in the brain tissue. L-DOPA is converted in the substantia nigra where it affects the doperminergic nitrostriatal tract which becomes extensively damaged in patients with Parkinsons disease.

L-DOPA is synthesised using the enzyme tyrosine-phenol lyase from *Erwinia herbicola* ATCC 21433 and developed by Ajinomoto Co. who produce around 250 tonnes per year, half of the worldwide L-DOPA demand. *E. herbicola* is added to a mixture of ammonium pyruvate and benzene 1,2-diol (catechol) to form L-DOPA (Shimizu *et al.*, 2002; figure 1.11).

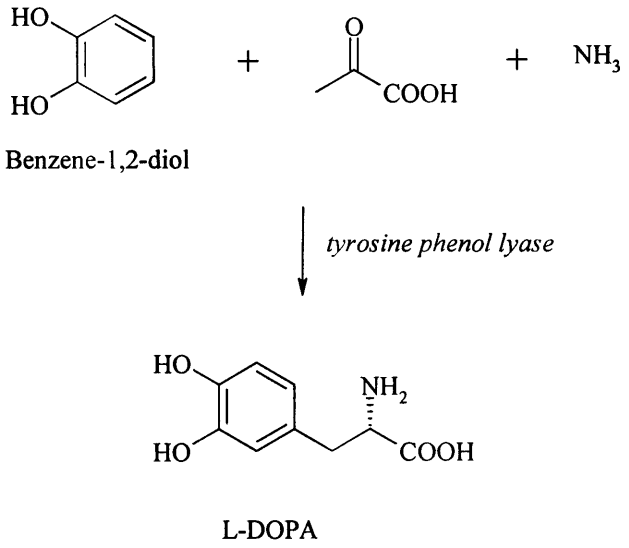


Figure 1.11: Conversion of ammonium pyruvate and catechol into L-DOPA via biocatalytic step using tyrosine-phenol lyase isolated from *Erwinia herbicola* by Ajinomoto Co.

Glipizide is a sulfonylurea and is used in the treatment of Type II diabetes. Glipizide has a direct effect on the β cells in the pancreas stimulating insulin release.

The precursor required in the synthesis of Glipizide is 2,5-dimethylpyrazine which is acted upon by *Pseudomonas putida* ATCC 33015 which oxidises a methyl group to a carboxylic acid group. This then undergoes further synthetic steps to yield Glipizide, (Zaks *et al.*, 1997; figure 1.12).

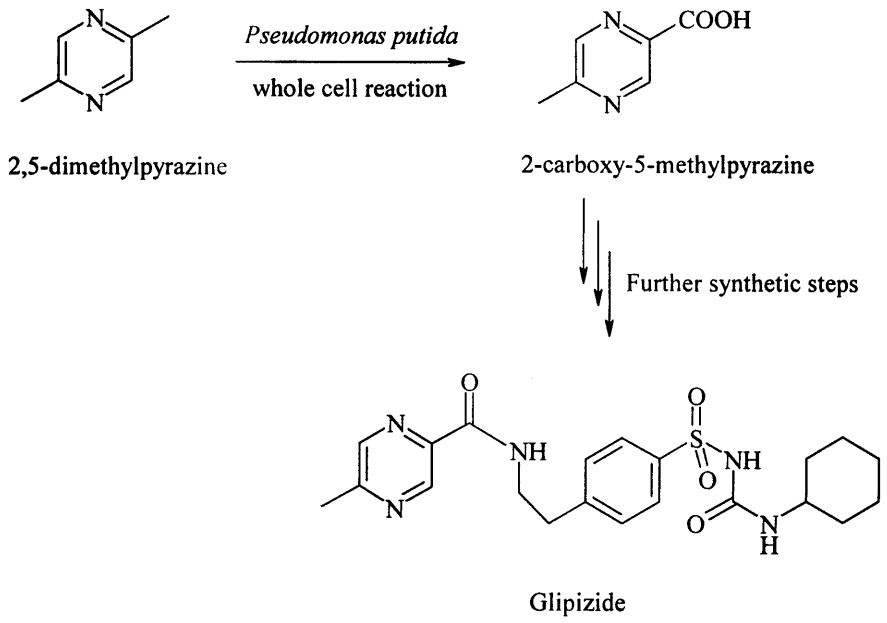


Figure 1.12: Synthesis of Glipizide using *P. putida*

More recently the novel use of enzymes in the production of Ziagen® (abacavir sulfate) by Glaxo Wellcome has been reported. Ziagen is a nucleotide inhibitor targeted at HIV control, whose key intermediate is a (-)-γ-lactam, (-)-2-azabicyclo[2.2.1]hept-5-en-3-one, which is produced by ChiroTech (Cambridge) via a biocatalytic resolution (Taylor *et al*, 1999). A racemic lactam, (±)-2-azabicyclo[2.2.1]hept-5-en-3-one, is resolved during a biotransformation using a recombinant (+)-γ-lactamase enzyme from *Comomonas acidovorans* over expressed in *E. coli*. The enzyme is highly selective for the hydrolysis of the (+)-γ-lactam leaving behind the required (-)-γ-lactam. From this stage further chemical synthesis can be carried out to yield abacavir sulfate, figure1.13.

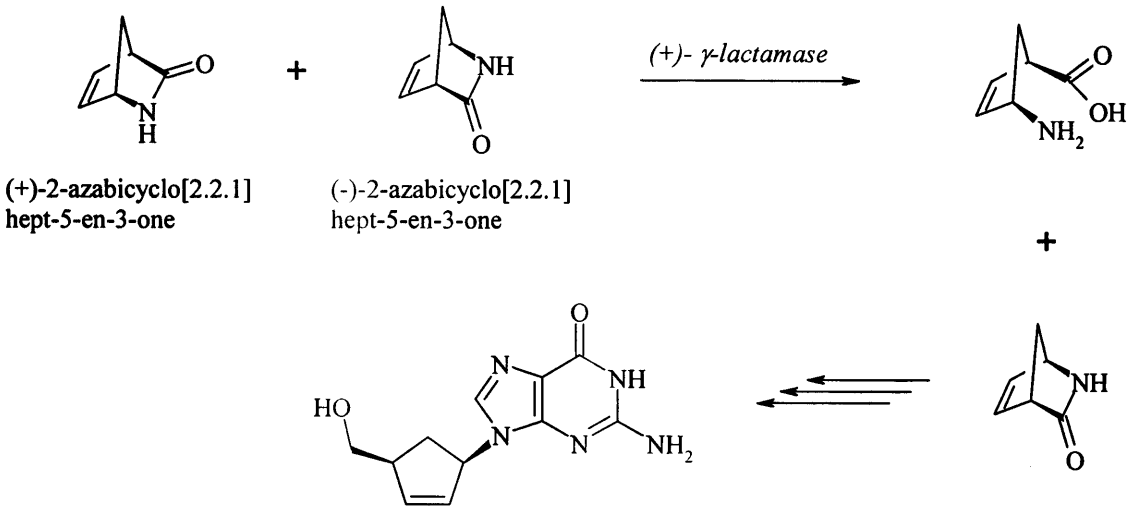


Figure 1.13: Biocatalytic resolution of (±)-2-azabicyclo[2.2.1]hept-5-en-3-one to the required (-)-2-azabicyclo[2.2.1]hept-5-en-3-one lactam by *C. acidovorans* (+)-γ-lactamase followed by several chemical steps to yield abacavir.

Undoubtedly, the chemical industry has benefited by incorporating biochemical techniques into production of useful precursors for large scale chemical manufacture. In 1999 Celgro utilised enzymes in the synthesis of two herbicides, metolachor and dimethenamide. The synthesis used an enzyme-catalysed transamination to produce a precursor of the herbicides (Matcham *et al.*, 1999; figure 1.14).

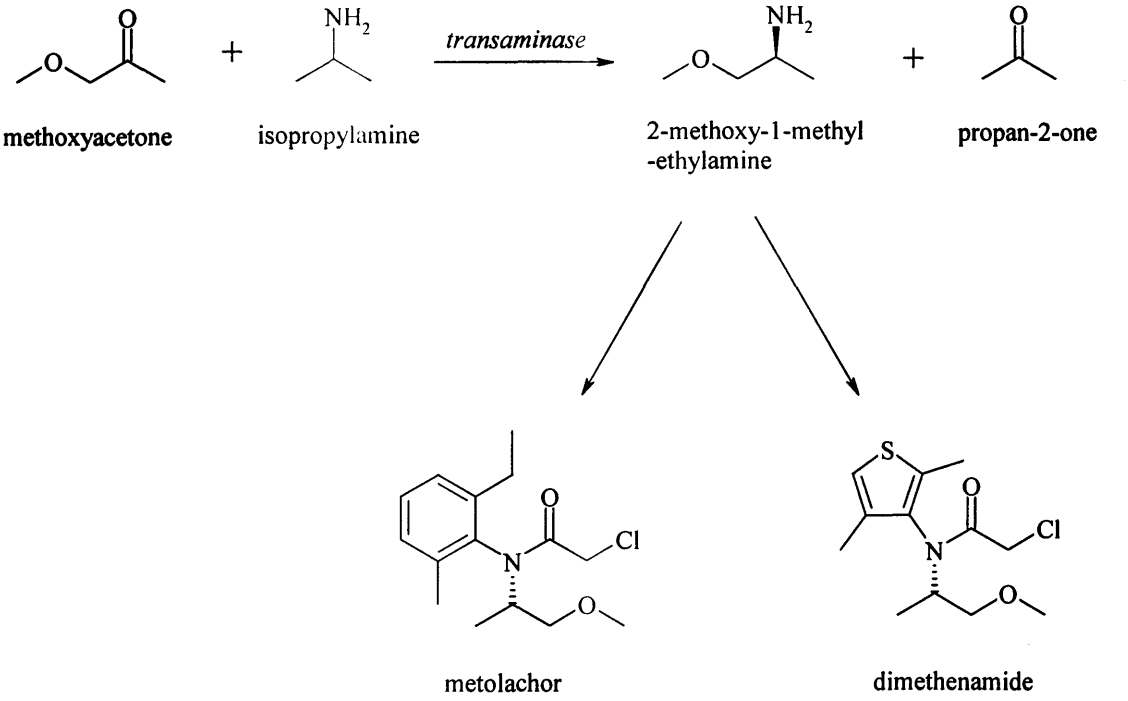
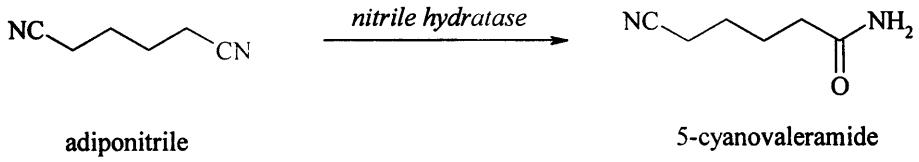


Figure 1.14: Scheme showing the conversion of methoxyacetone and isopropylamine by transamination to yield metolachor and dimethenaminde.

DuPont have used a similar approach in the manufacture of an intermediate used in the production of a new herbicide. The process was developed as an alternative to chemical hydration which produced many by products. The new biocatalytic process is more efficient and cleaner producing 3150 Kg of product per Kg of catalyst. The process uses a nitrile hydratase isolated from *Pseudomonas chlororaphis*. This is immobilized on calcium alginate beads allowing it to be effectively recycled (Hann *et al.*, 1999; figure 1.15).

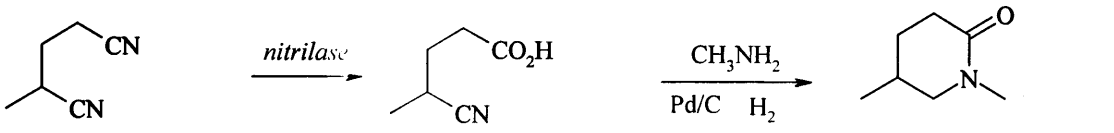


adiponitrile 5-cyanovaleramide

Figure 1.15: Scheme showing the conversion of adiponitrile to 5-cyanovaleramide using nitrile hydratase from *P. chlororaphis*

Recently DuPont have harnessed the capabilities of nitrilases which hydrolyse nitriles through to the corresponding carboxylic acids. A nitrilase has been isolated from *Acidovorax facilis* and have used it to produce 1,5-dimethyl-2-piperidine, a useful solvent similar to N-methyl-2-piperidine which has applications in electronics and coatings.

The process converts 2-methylglutaronitrile to 4-cyanopentonic acid which undergoes hydrogenation in the presence of methylamine to yield the product. Overall the process is highly efficient with the generation of 1 tonne of product using 1 Kg of dry cells with 98% selectivity (Cooling *et al,* 2001; figure 1.16).



2-methylglutaronitrile 4-cyanopentonic acid 1,5-dimethyl-2-methylene -piperidine

Figure 1.16: Conversion of 2-methylglutaronitile by a nitrilase from *A. facilis*. Further hydrogenation of 4-cyanopentanoic acid yields 1,5-dimethyl-2-methylene-piperidine

1.2.5 Multistep reactions

In the previous sections the use of biocatalysts in the pharmaceutical and chemical industry has been highlighted. However, most if not all reactions only use biocatalysts for one step in the reaction pathway which is often followed by further chemical synthesis. Often only one bioconversion is possible due to the nature of the host organism with regards to stability (enzyme over expression). It would therefore be highly advantageous to either utilise a single microbial organism to provide a multistep bioconversion or multiple biocatalysts in a single pot synthesis providing a high efficiency method whilst overcoming regio and stereoselective problems associated with organic synthesis.

One such example using two biocatalysts in a single pot reaction is the conversion of α-keto esters to a hydroxy acid via a keto-acid (Gibbs *et al.*, 1999 and Sutherland *et al.*, 1998). In this reaction a lipase from *Candida rugosa* hydrolyses the α-keto esters to their corresponding α-keto acids which are then converted in to hydroxyl acid using lactate dehydrogenase isolated from *Bacillus stearothermophilus* or *Staphylococcus epidermis*, figure 1.17.

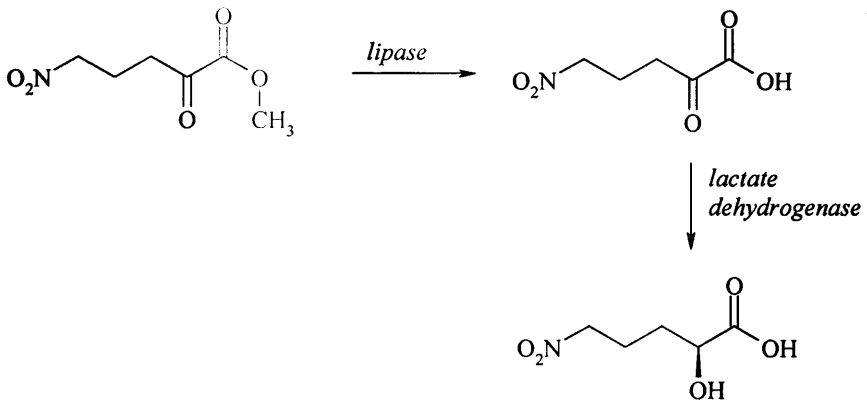


Figure 1.17: Conversion of α-keto ester to hydroxy acid via a keto-acid using a lipase and lactate dehydrogenase in a single pot reaction.

However, the above multi bioconversion relies on using free enzymes in an aqueous environment which are not always stable and require the use of cofactors to drive the reaction. Therefore the use of a single microorganism which is capable of performing multistep reactions would remove the requirement of expensive cofactors and provide a more robust and stable operating environment. The main components of a multistep bioconversion are the substrate(s), products(s), pathway intermediates, enzymes involved and the host organism. In order to qualify as a potentially viable process these components should display the following abilities (Marshall *et al.*, 1995);

- The host must contain specific uptake mechanisms to ensure the substrate is available for pathway enzymes
- The appropriate metabolic pathway enzymes must act on the substrate
- The product must accumulate extracellularly and in a high concentration to enable efficient product recovery and not be metabolised further
- The products and intermediates must be stable in the reaction medium
- Cofactors must be recycled by the host organism
- The enzyme activity must not decrease during process conditions or storage

With the advancement of recombinant DNA technology it is now possible to selectively clone useful enzymes from many host organisms and create a single host organism that displays specific biocatalytic activity thus enabling multistep biocatalysis. One such system is that based on the TOL plasmid pathway. This TOL plasmid was first discovered by Williams and co workers in 1974 in a mutant strain of *Pseudomonas putida* (*arvilla*) mt-2 (Williams *et al.*, 1974). This mutant strain shows the ability to metabolise catechol via *meta*-cleavage and is only displayed on the plasmid and is non-specific with regards to induction and catalysis, whereas the wild type strain has the ability to metabolise benzoate via *ortho*-cleavage using benzoate specific pathway enzymes and is encoded on the chromosome, figure 1.18.

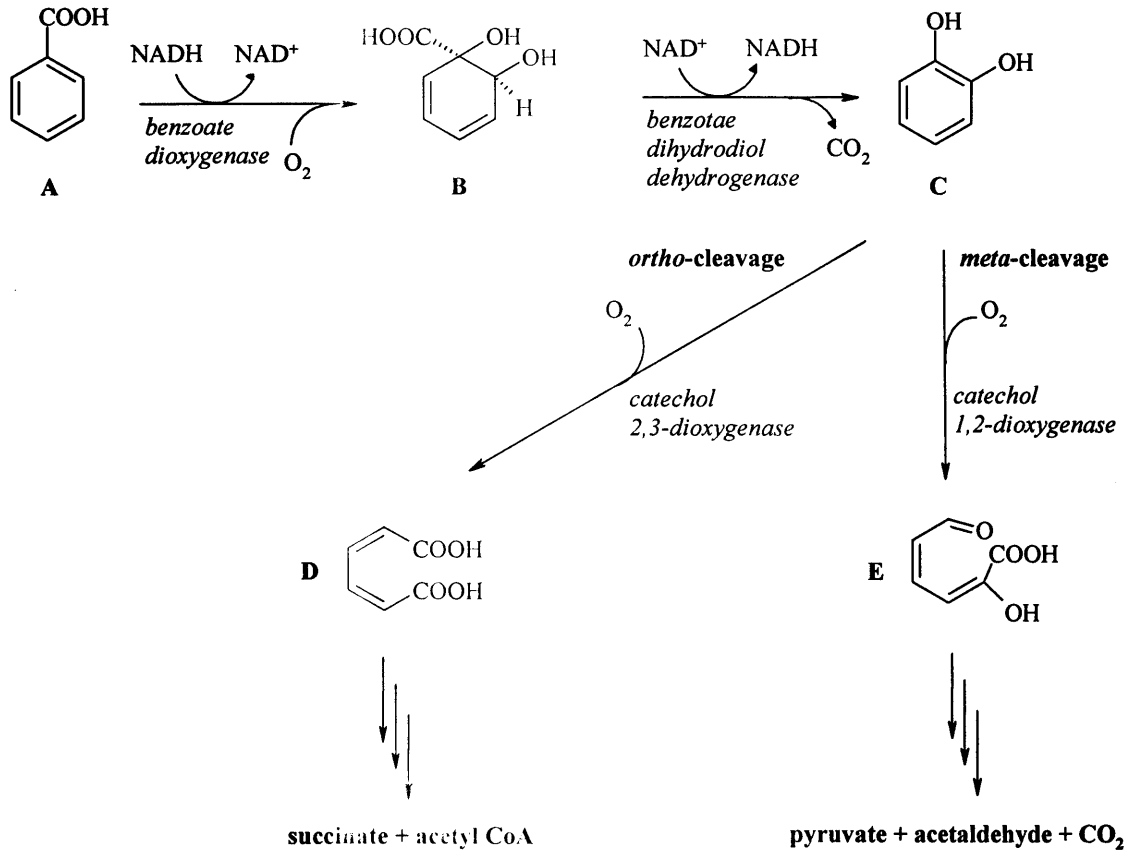


Figure 1.18: ***meta*** **and** ***ortho*** **cleavage pathways in** *P. putida.* **A- benzoate B- benzoate dihydrodiol C- catechol D-** ***cis*** **muconic acid E- 2-hydroxymuconic semialdehyde**

With recent developments in DNA technology Bühler and co workers have since isolated and cloned a xylene monooxygenase, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase from this mutant *P. putida* strain and successfully over expressed it in *E. coli* JM101 (Bühler *et al.*, 2000). It has been shown that this xylene monooxygenase catalyses the multistep oxidation of toluene and pseudocumene via the corresponding alcohol and aldehyde to the benzoic acids, figure 1.19.

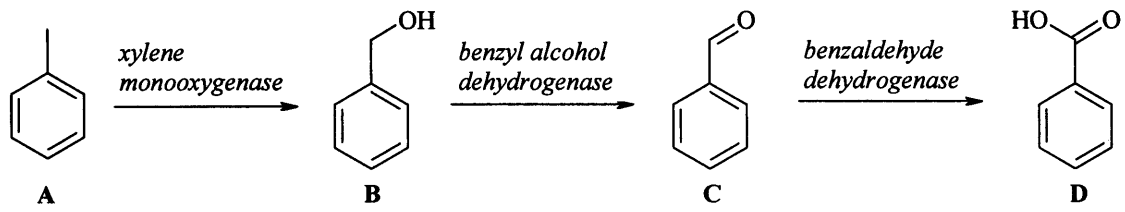


Figure 1.19: Multistep pathway of recombinant *E. coli* JM101 displaying xylene monooxygenase activity A- toluene B- benzyl alcohol C- benzaldehyde D- benzoic acid

The use of multistep biocatalysis together with DNA technology holds huge potential for the future as currently known biocatalysts can be cloned and over expressed into a whole cell microorganism providing synthesis of regio- and stereo- pure products. However, finding and exploiting naturally occurring biocatalysts which show the ability to perform multistep reactions is highly desirable as often isolating and combining genomes from multi organisms is time consuming, expensive and not always successful.

As demand grows to find new biocatalysts it is necessary to explore new chemistries such as carbon-carbon bond formation and oxidative biocatalysis. Oxidation reactions play a huge role in chemical synthesis as previously discussed but have limitations as regards to enantioselectivity. Over the last decade interest in oxygenase enzymes has increased and this group is commercially important for performing stereoselective reactions which by classical chemical synthesis are difficult to achieve.

1.3 Oxidoreductase classification

1.3.1 Introduction

As previously discussed in section 1.2.1 these enzymes involve the transfer of hydrogen or oxygen atoms between molecules and includes enzymes such as the dehydrogenases, oxidases, oxygenases and peroxidases.
In this thesis the main focus will be on the oxygenases and their use in performing regio and stereoselective oxidations.

Oxygenases can be primarily classified into two groups; monooxygenases and dioxygenases (Walsh and Chen, 1988; Van Berkel and Muller, 1991). Monooxygenases, as the name implies, catalyse the breaking of the molecular oxygen bond inserting one oxygen atom into the substrate with the other undergoing reduction to form water. Dioxygenases insert two oxygen atoms in to the substrate, both of which are derived from the breaking of molecular oxygen, figure 1.20.

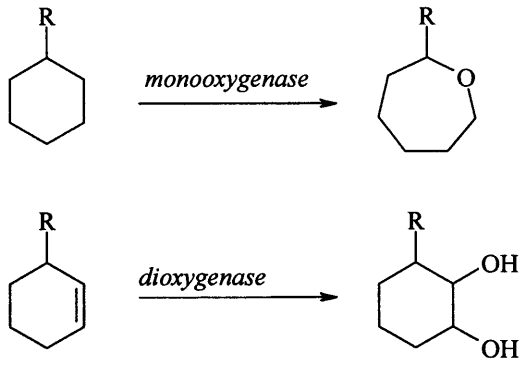


Figure 1.20: Scheme showing monooxygenase and a dioxygenase reaction

Monooxygenases can be further subdivided depending on the substrate used. They can be classed as Baeyer-Villiger monooxygenases where 4α-hydroperoxyflavin oxygenation of electrophilic or nucleophilic substrates occurs or hydroxylases where hydroxylation, the conversion of a carbon-hydrogen to a carbon-hydroxyl bond occurs.

A third variant are the luciferases which belong in this class of flavoproteins. Bacterial luciferase is a flavin dependent monooxygenase which catalyses the oxidation of flavin

mononucleotide ($FMNH_2$) and a long chain aliphatic aldehyde in a blue-green light emitting reaction, figure 1.21.

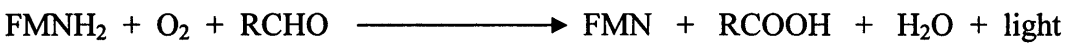
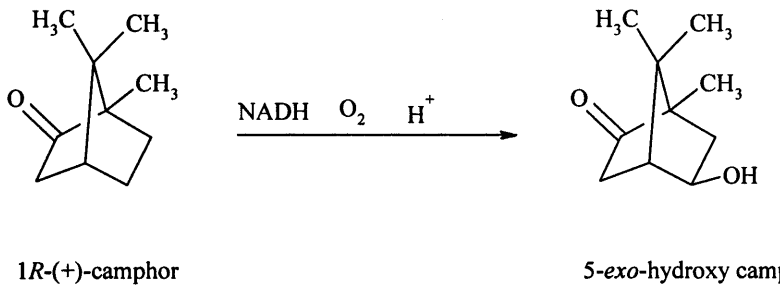


Figure 1.21: Scheme showing the bacterial luciferase reaction

The monooxygenase group also includes the P450 enzymes, often referred to as cytochrome P450. They make up a superfamily of haem-thiolate proteins (the haem iron fifth ligand is a thiolate group, typically of a cysteine residue) (NCIBUB, 1991) which are found in bacteria, fungi, plants and animals. The enzymes catalyze oxygen insertion into many different kinds of substrates, including natural steroids, fatty acids and foreign compounds where they serve as the enzymes for drug metabolism and detoxification of xenobiotic compounds prevalent in the environment.

P450 containing monooxygenase systems primarily fall into two major classes; bacterial/mitochondrial (class I) containing a P450, ferredoxin and a FAD-ferredoxin reductase (NADH-dependent). Microsomal (class II) are two component systems, usually membrane bound, which contain a NADPH-dependent diflavin reductase (FAD and FMN) and P450 (Nebert *et al.*, 1987). Alternatively, P450 containing systems can be classified according to the number of their protein components (Degtyarenko *et al.*, 1993). The most studied P450 is the camphor hydroxylase isolated from *Pseudomonas putida* catalysing the regio and stereospecific hydroxylation of 1(R)-(+)- camphor to 5-*exo*-hydroxy camphor (Poulos *et al.*, 1986; figure 1.22)



1*R*-(+)-camphor

5-*exo*-hydroxy camphor

Figure 1.22: Regio and stereospecific hydroxylation of 1(*R*)-(+)- camphor to 5-*exo*-hydroxy camphor by camphor hydroxylase isolated from *P. putida*

However, two new classes of P450s have since been discovered, class III P450s, such as P450 BM3 from *Bacillus megaterium* (Narhi *et al.*, 1986) containing the same cofactors as the class II P450s but they are soluble and fused into one continuous polypeptide and more recently class IV P450s which are one component enzymes but containing an NADH-dependent, FMN-containing reductase and ferredoxin fused to the heme domain and also soluble which has been discovered in *Rhodococcus* (Roberts *et al.*, 2002).

In this thesis the central enzyme of interest in *C. echinulata* is the CeBVMO, as discussed in thesis aims and objectives in section 1.4. This enzyme will therefore become the main focus in the next section where the Baeyer-Villiger reaction will be discussed in further detail along with the operation of the CeBVMO.

1.3.2 The Baeyer-Villiger reaction

In 1899 Adolf Von Baeyer and Victor Villiger (Baeyer and Villiger, 1899) discovered that cyclic ketones are converted to lactones upon reaction with per-acids such as Caro's acid (permonosulfuric acid; HO_2SO_3H) figure 1.23.

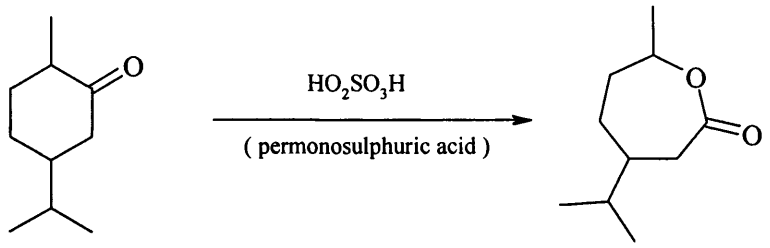


Figure 1.23: Conversion of cyclic ketone in to cyclic lactone using permonosulfuric acid

Since this discovery many other variations of this reaction have been derived. Currently the most popular chemical oxidants are *meta*-chloroperoxybenzoic acid, peroxyacetic acid and a mixture of an organic acid and hydrogen peroxide which forms the peroxyacid in situ. Currently the accepted mechanism for the chemical Baeyer-Villiger oxidation is that first proposed by Criegee in 1948 (Criegee, 1948). Reversible addition of the peracid terminal oxygen to the carbonyl yields a tetrahedral species that undergoes concerted rearrangement and fragmentation to products, figure 1.24.

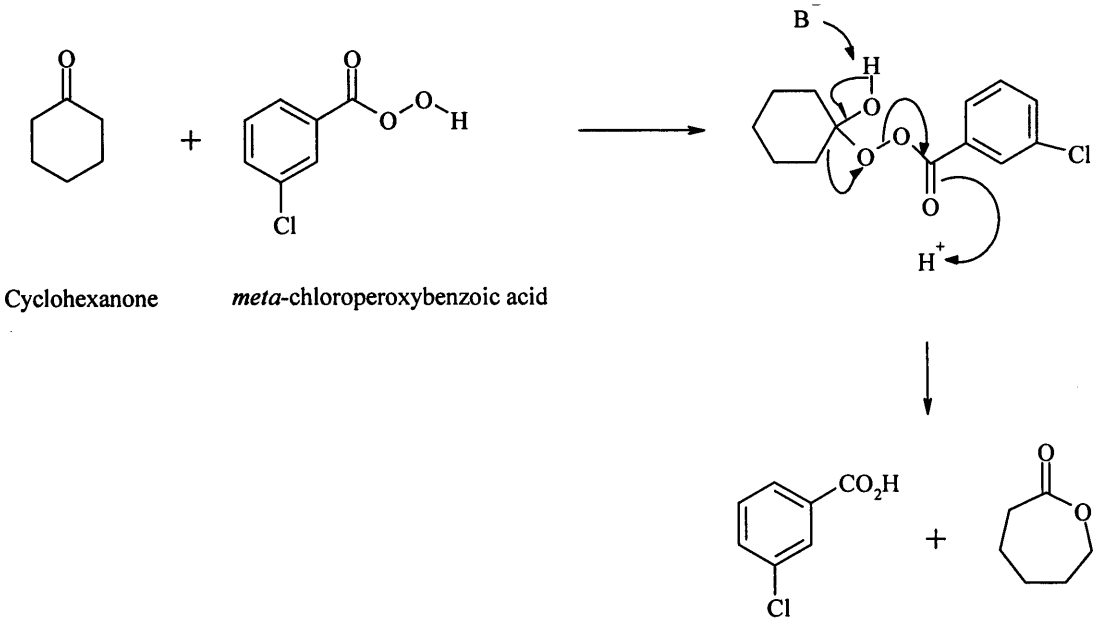


Figure 1.24: Baeyer-Villiger oxidation reaction mechanism

However, these reagents are unstable, especially *meta*-chloroperoxybenzoic acid which, in its liquid form is explosive. Therefore more stable oxidants such as bis(trimethylsilyl)peroxide and magnesium monoperoxyphthalate are now used.

Many of these reagents do not act specifically and they may also attack other non-functional groups such as alkenes, amines, phosphines and sulfoxides. The products are never stereoselectively pure but an enantiomeric mixture. To overcome this problem recent development using metal based catalysts to perform metal-assisted asymmetric Baeyer-Villiger oxidations have been employed producing enantioenriched lactones based on transition metal catalysis. The use of copper and platinum as chiral catalysts has been extensively studied (Bölm *et al.*, 1994,1995 and 1997; Gusso *et al.*, 1994 and Paneghetti *et al.*, 1999) and it has been found that their use leads to highly enantioselective reactions with a broad range of substrates, particularly substituted cyclobutanones and cyclohexanones, figure 1.25.

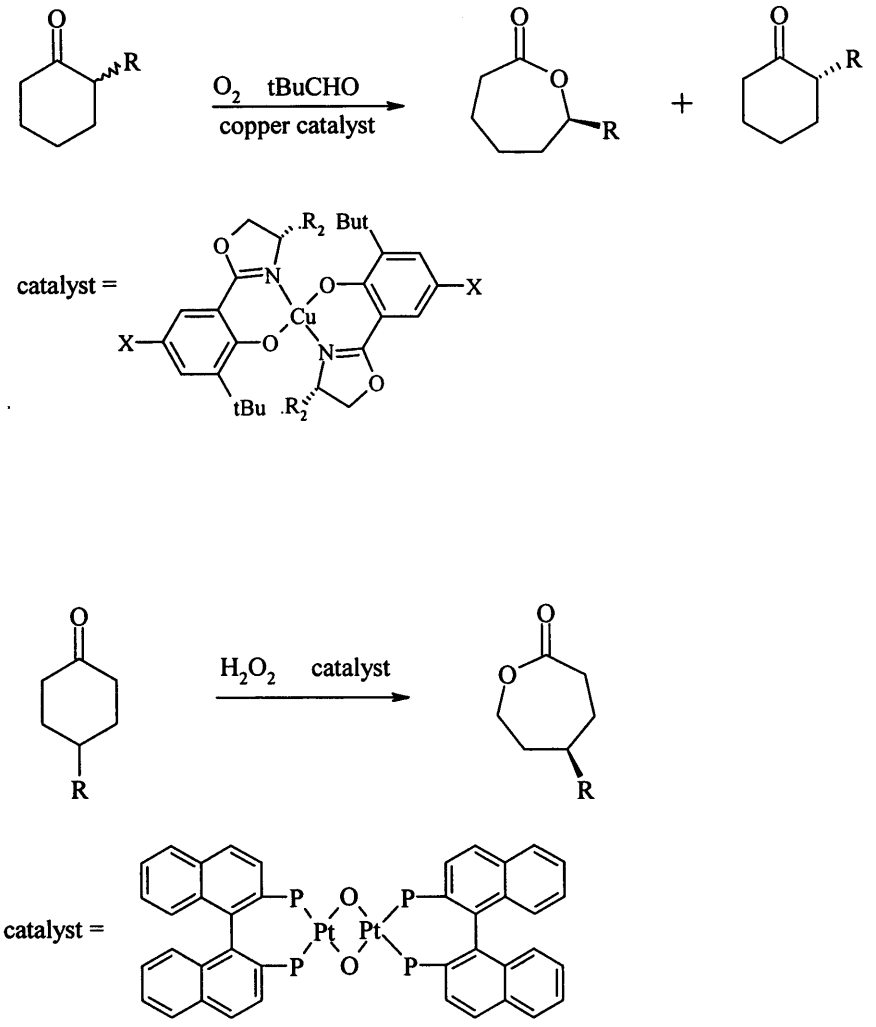


Figure 1.25: Conversion of substituted cyclohexanones in to lactones using chiral copper and platinum metal catalysts where X= NO_2 or H and R_2= $_t$-Bu or $_i$Pi

However, the use of metal based catalysts has had limited success and the use of biocatalysts to perform the Baeyer-Villiger reaction is highly appealing. Over the past ten years several Baeyer-Villiger monooxygenase enzymes (BVMO) have been found (Alphand *et al.*, 2001; Mihovilovic *et al.*, 2002; Stewart *et al.*, 1998 and Willetts 1997). These BVMO are extremely attractive for synthetic applications due to the nucleophilic oxygenation of the ketones and electrophilic oxygenation of various heteroatoms which can proceed with high enantioselectivity when using racemic substrates (kinetic resolution) or enantiotoposelectivity using achiral substrates (asymmetric synthesis), figure 1.26.

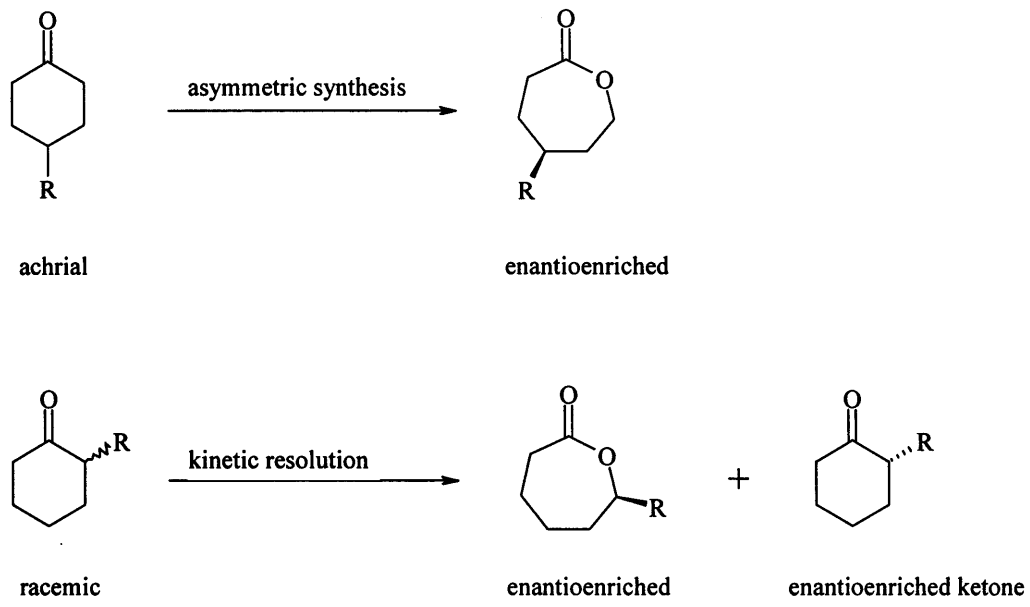


Figure 1.26: Asymmetric synthesis and kinetic resolution using Bayer-Villiger monooxygenases

1.3.3 Baeyer-Villiger Monooxygenase reaction

The first one step enzymatic Baeyer-Villiger oxidation was carried out by Shaw in 1966 (Shaw, 1966) using *Pseudomonas oleovorans* NCIMB 6576 and (±)-2 heptylcyclopentanone as the substrate to yield δ-heptyl valerolactone, figure 1.27.

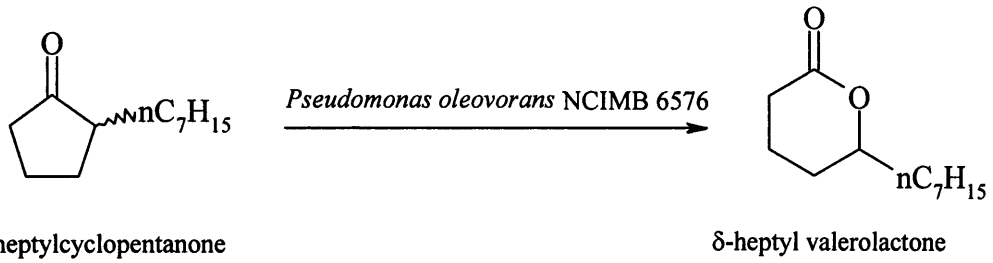


Figure 1.27: The BVMO catalysed formation of δ-heptyl valerolactone

Since this first biotransformation was carried out many other species of bacteria and fungus have been found to display BVMO activities against a wide variety of substrates which are capable of performing stereospecific Baeyer-Villiger oxidations with enantiopure products (Roberts and Wan, 1998).

The most studied of these BVMOs is the cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* NCIMB 9871 isolated in 1975 (Trudgill *et al.*, 1975). It is a 60 KDa Type 1 monomeric monooxygenase containing a FAD prosthetic group and is oxygen and NADPH dependent. It allows the growth of bacteria on cyclohexanol or cyclohexanone as the sole carbon source, figure 1.28. The monooxygenase catalyses the conversion of cyclohexanone to ε-caprolactone by the classical Baeyer-Villiger rearrangement.

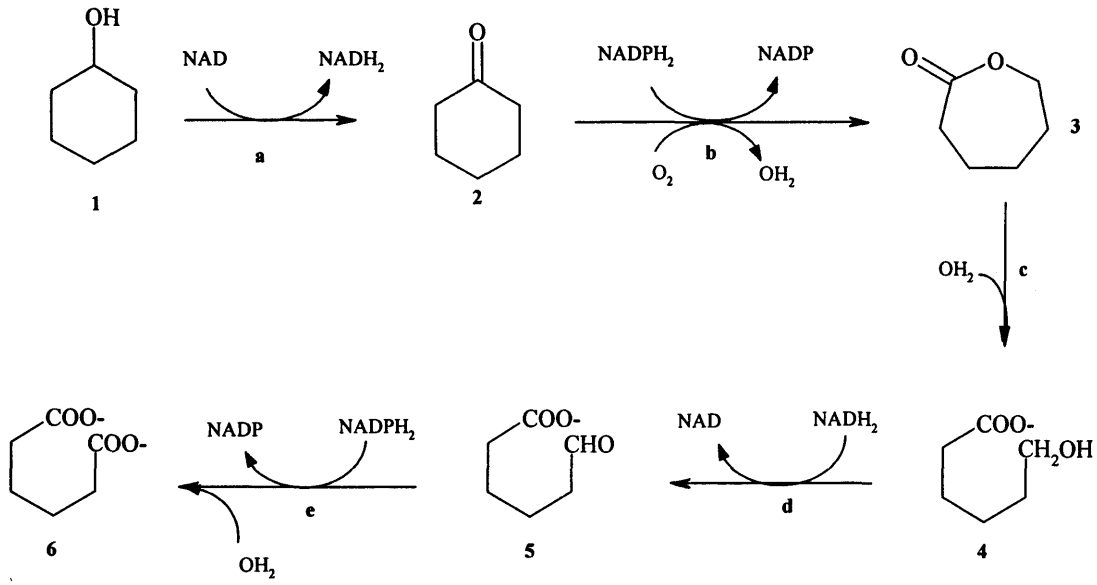


Figure 1.28: Degradation pathway of cyclohexanol to adipate by *A. calcoaceticus* NCIMB 9871 showing in particular the role of the cyclohexanone monooxygenase enzyme (b) and the lactonase enzyme (c).
1:- cyclohexanol; 2:- cyclohexanone; 3:- 1-oxa-2-oxocycloheptane; 4:- 6-hydroxyhexanoate; 5:- 6-oxohexanoate; 6:- adipate.
a:- cyclohexanol dehydrogenase; b:- cyclohexanone 1,2-monooxygenase; c:- 1-oxa-2-oxocycloheptane lactonase; d:- 6-hydroxyhexanoate dehydrogenase; e:- 6-oxohexanoate dehydrogenase. From (Watts *et al*, 2002)

To date, monooxygenases can be classified in two ways, either from a biological view or chemical standpoint.

Biologically there are two types of BVMO;

Type 1 monooxygenases are homogeneous enzymes that are either monomeric or as an oligomer, with up to four identical protein subunits. Most Type 1 monooxygenases require NADPH to supply the external electrons for the reaction, and FAD as the flavin coenzyme. Although most Type 1 monooxygenases utilise FAD there are exceptions, for example the cyclohexanone monooxygenase from *Xanthobacter* sp. which is NADPH and FMN dependent (Tanner *et al.*, 2000). Some common examples of Type 1 monooxygenases are shown in table 1.3.

Type 2 monooxygenases have been found to be more complex. All are heterogeneous consisting of both oxygenating and dehydrogenase subunits and requiring NADH and FMN as cofactors. To date very few examples exist and mechanisms are not known. Known examples of Type 2 monooxygenases are shown in table 1.4.

From a chemical view the classification of monooxygenases is reversed compared to biological classification. Type 1 monooxygenases are known as MO2 (FAD and NADPH dependent) and Type 2 monooxygenases are known as MO1 (FMN and NADH dependent).

Table 1.3 Type 1 monooxygenase enzymes

Enzyme/ Source	Subunit Structure	Nicotinamide cofactor specificity	Native MW (kDa)	Mol flavin/ Mol subunit	Reference
Cyclohexanone Monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871	Monomer	NADPH	59	1 FAD	Donoghue *et al.*, 1976
Cyclohexanone Monooxygenase from *Norcardia* sp. CL1	Monomer	NADPH	53	1 FAD	Donoghue, Norris *et al.*, 1976
Cyclopenatanone Monooxygenase from *Pseudomonas* NCIMB 9872	3-4 identical units	NADPH	200	1 FAD	Griffin and Trudgill, 1975
Steroid Monooxygenase from *Cylindrocarpon radicicola* ATCC 11011	Dimer (Subunits identical)	NADPH	115	1 FAD	Itagaki, 1986
2-oxo-Δ^3-4,5,5-trimethylcyclopentenyl acetyl CoA monooxygenase from *P. putida* NCIMB 10007 (MO2)	Dimer (Subunits identical)	NADPH	106	1 FAD	Ougham *et al.*, 1983
2-tridecanone monooxygenase from *P. cepacia*	Dimer	NADPH	123	1 FAD	Britton and Markovetz, 1977
Cyclohexanone monooxygenase from *Xanthobacter* sp.	Monomer	NADPH	50	1 FMN	Trower *et al.*, 1989
Arylketone monooxygenase	Monomer	NADPH	70	1 FAD	Tanner *et al.*, 2000
4-hydroxyacetophenone monooxygenase from *P. fluorescens*	Dimer	NADPH	140	1 FAD	Kamerbeek *et al.*, 2001

Table 1.4 Type 2 monooxygenase enzymes

Enzyme/ Source	Subunit Structure	Nicotinamide cofactor specificity	Native MW (kDa)	Mol flavin / mol subunit	Reference
2,5-diketocamphane 1,2, monooxygenase from *P. putida* NCIMB 10007	4 protein subunits NADH dehydrogenase and dimeric oxygenating component	NADH	2× 39 kDa for the oxygenating subunits; 36-40 kDa for the dehydrogenase	1 FMN	Taylor and Trudgill, 1986
3,6-diketocamphane 1,6-monooxygenase from *P. putida* NCIMB 10007	4 protein subunits NADH dehydrogenase and dimeric oxygenating component	NADH	2× 39 kDa for the oxygenating subunits; 36-40 kDa for the dehydrogenase	1 FMN	Jones *et al.*, 1993
2-oxygenase from *P. flava* ACM 1742	4 protein subunits NADH dehydrogenase and dimeric oxygenating component	NADH	2× 35-40 kDa for the oxygenating subunit; dehydrogenase MW not reported	1 FMN	Williams, 1991

As discussed previously the Type 1 monooxygenase requires NADPH and FAD to carry out the reaction. It has been found by Walsh and co-workers that the FAD forms the key oxygenating intermediate (Walsh *et al.*, 1988). The oxidised flavin found in the resting form of the enzyme is reduced by hydride transfer from NADPH. Molecular oxygen binds to the carbocation forming 4a-hydroperoxide. The terminal oxygen of this intermediate is thought to add to the carbonyl group of the ketone forming a tetrahedral intermediate similar to that of the Criegee intermediate. This intermediate then undergoes fragmentation to yield the lactone and the 4a-hydroxyflavin which then undergoes dehydration to regenerate the oxidised flavin, figure 1.29.

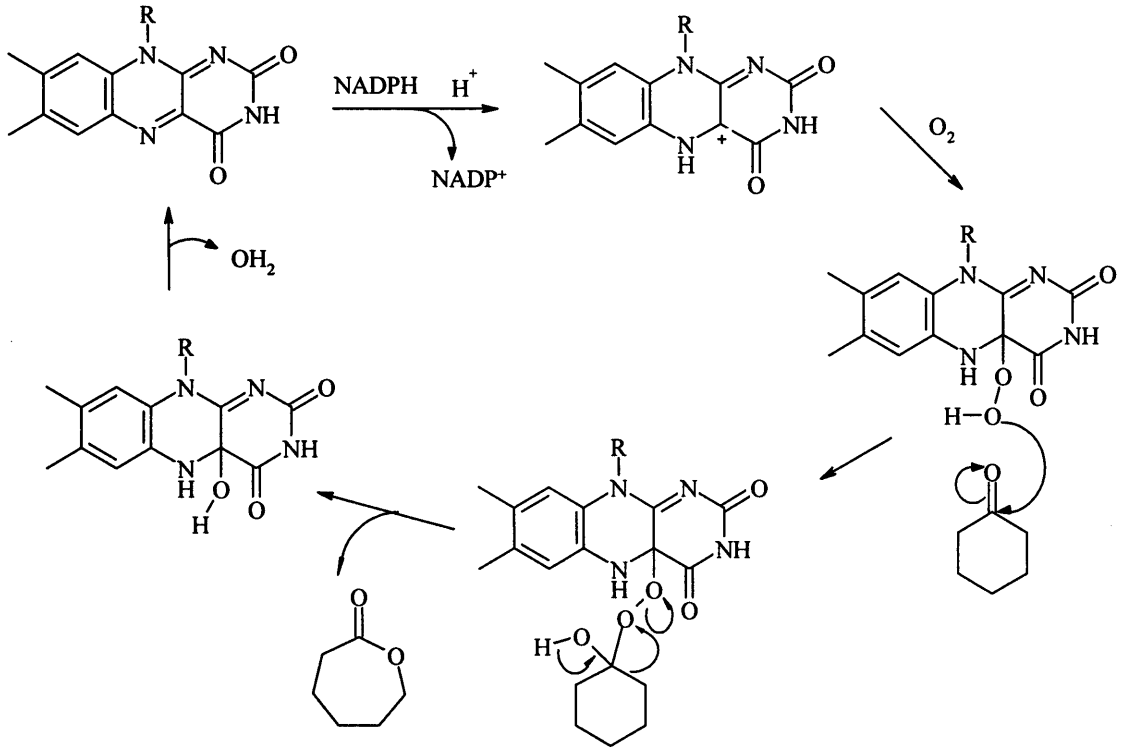


Figure 1.29: Baeyer-Villger monooxygenase scheme utilising FAD as the key oxygenating species. FAD is reduced by NADPH followed by oxygenation leading to 4a-hydroperoxide formation. This hydroperoxide attacks the ketone carbonyl group forming a Criegee intermediate. Fragmentation results in lactone formation and generation of 4a-hydroxyflavin which then undergoes dehydration to regenerate the oxidised flavin.

Over the last 10 years Baeyer-Villiger monooxygenases from a broad spectrum of microorganisms have been employed as chiral catalysts to perform many regioselective Baeyer-Villiger oxidations of racemic or prochiral ketones and chiral lactones. The biotransformations may be carried out as whole cell reactions, using the partially purified protein or a pure enzyme. To date around thirty BVMO enzymes have been isolated, mostly from bacterial sources, although studies into fungal strains have also yielded a broad and varied collection of BVMO activities (Carnell and Willetts, 1992), some examples are shown in table 1.5.

Table 1.5: Examples of microorganisms containing Bayer-Villiger type monooxygenases. Abbreviations: B-bacterial, F-fungal U-unknown/ not determine

Enzyme	Source	Species	Cofactor	Substrate(s)	Reference
			FMN/FAD/ NADH/NADPH		
Arylketone monooxygenase	B	*P. putida* JD1	FAD NADPH	4-hydroxy-acetophenone	Tanner *et al*, 2000
2,5-Diketo-camphane 1,2-monooxygenase	B	*P. putida* NCIMB 100007	FMN NADH	2,5-Diketo-camphane	Kelly *et al*, 1998
3,6-Diketo-camphane 1,6-monooxygenase	B	*P.* putida NCIMB 100007	FMN NADH	3,6-Diketo-camphane	Kelly *et al*, 1998
Cyclohexanone monooxygenase	B	*Xanthbacter* sp.	FAD NADPH	Cyclohexanone	Trower *et al*, 1989
Steroid monooxygenase	B	*Rhodococcus erythropolis*	FAD NADPH	Progesterone, 11α- and 11β-hydroxyprogesterone, C_{21}-20-ketosteroid	Miyamoto *et al*, 1995 and Morii *et al*, 1999
Monocyclic monoterpene ketone monooxygenase	B	*R. erythropolis* DCL 14	FAD NADPH	Monocyclic monterpene ketones 1-hydroxy-2-oxolimonene	Van Der Werf, 2000
Cyclopentanone 1,2-mono-oxygenase	B	*Pseudomonas* NCIMB 9872	FAD NADPH	Cyclopentanone	Griffin and Trudgill, 1975
Cyclohenxanone 1,2-mono-oxygenase	B	*Nocardia globerula* CL1	FAD NADPH	Cyclopentanone, Cyclohexanone	Donoghue *et al*, 1976
Cyclohexanone 1,2-mono-oxygenase	B	*Acinetobacter* NCIMB 9871	FAD NADPH	Cyclohexanone	Donoghue *et al*, 1976
Baeyer-Villiger monooxygenase	B	*P. cepacia*	FAD NADPH	2-Tridecanone	Kelly, Wan, *et al*, 1998
4-hydroxy-acetophenone monooxygenase (HAPMO)	B	*P. fluorescens* ACB	FAD NADPH	4-hydroxy-acetophenone	Kamerbeek *et al*, 2001
Steroid monooxygenase	F	*Cylindrocarpon radiciola* ATCC 11011	FAD NADPH	C_{21}-20-ketosteroid	Itagaki, 1986
Baeyer-Villiger monooxygenase	F	*Drechslera australiensis*	U	Bicyclo[3.2.0]-hept-2-en-6-one	Carnell *et al*, 1992

Baeyer-Villiger monooxygenase	F	*Curvularia lunata* NRRL 2380	U	Bicyclo[3.2.0]-hept-2-en-6-one	Carnell *et al* 1992
Baeyer-Villiger monooxygenase	F	*Cur. Senegalensis*	U	Bicyclo[3.2.0]-hept-2-en-6-one	Carnell *et al*, 1990
Baeyer-Villiger monooxygenase	F	*Cyl. destructans*	U	Bicyclic ketones	Konigs-berger *et al*, 1990
Baeyer-Villiger monooxygenase	F	*Beauveria bassiana*	U	4-(4-hydroxy-phenol)butan-2-one	Fuganti *et al*, 1996
Baeyer-Villiger monooxygenase	F	*Cunninghamella echinulata* NRRL 3655	U	Bicyclo[3.2.0]-hept-2-en-6-one , 3-phenyl-cyclobutanone	Alphand and Furstoss, 2000

As briefly mentioned earlier in section 1.3.1, the use of monooxygenases to perform the Baeyer-Villiger reaction provides a powerful method of producing enantiopure lactones along with their ability to transform a large variety of non-natural substrates with high regio and enantioselectivity. It is possible to summarise the abilities of enzymes when presented with a racemic ketone substrate compared to that of chemical oxidation in the following way, figure 1.30.

1 $K_{(+/-)} \longrightarrow L_{1\,(+/-)}$

Chemical synthesis: Regioselective and enantiodivergent

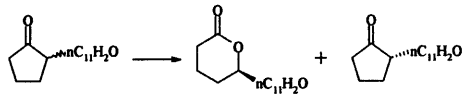
2 $K_{(+)} \longrightarrow L_{1(+)}\,L_{2(+)}$

$K_{(-)} \longrightarrow L_{1(-)}\,L_{2\,(-)}$

Enzymatic synthesis: Regiodivergent and enantiodivergent
Example: CHMO from *A. calcoaceticus*
Process issues: End products must be separated by additional chromatographic step

3 $K_{(+)} \longrightarrow L_{1\,(+)}$

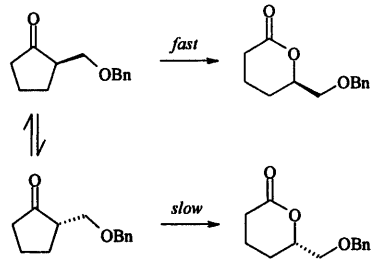
$K_{(-)} \not\longrightarrow L_{2\,(-)}$



Enzymatic synthesis: Regioselective and enantioselective. Conversion of $K_1 \rightarrow L_1$ favoured
Example: CHMO from *A. calcoaceticus* using bicyclo[2.2.1]heptan-2-one (10:1 ratio observed (Abril *et al,* 1989)
Process issues: Chromatographic separation of $K_{(-)}$ necessary from lactone

4 $K_{(+)} \longrightarrow L_{1(+)}$

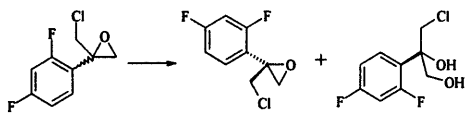
$K_{(-)}$



Enzymatic synthesis: Dynamic Kinetic Resolution, regioselective and enantioselective
Example: Conversion 2-benzyloxymethyl-cyclopentanone using *E. coli* TOP10 pQR239 (Berezina *et al.*, 2002)
Process issues: Lactone produced is single regioisomer

5 $K_{(+)} \longrightarrow L_{1(+)}$

$K_{(-)}$



Enzymatic synthesis: Convergent reaction regioselective and / or enantiodivergent
Example: Biocatalysed hydrolytic kinetic resolution. Conversion of racemic chlor-epoxide using *Aspergillus niger* (Monfort *et al.*, 2002)
Process issues: Lactone produced is single regioisomer, separation of hydrolysed product necessary

Figure 1.30: Scheme showing possible conversions of racemic starting substrate

BVMOs have enormous potential with regards to selectivity and the bio-oxidation of bicyclic ketones that display cyclobutanone structural motifs which are convenient substrates for organisms displaying BVMO activity and the use of bicyclo[3.2.0]hept-2-en-6-one ketone has become one of the "benchmark" reactions for BVMO performance.

Racemic compounds are transformed in a resolution process into two types of regioisomeric lactones, figure 1.31. The expected lactone, termed "normal", is generated by migration of the more substituted carbon atom, the corresponding lactone, termed "abnormal" or "enzyme induced" is formed by migration of the less substituted carbon atom. Each lactone product is derived from one enatiomeric substrate.

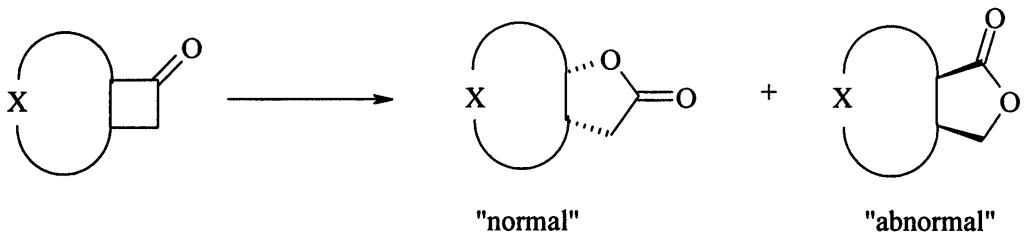
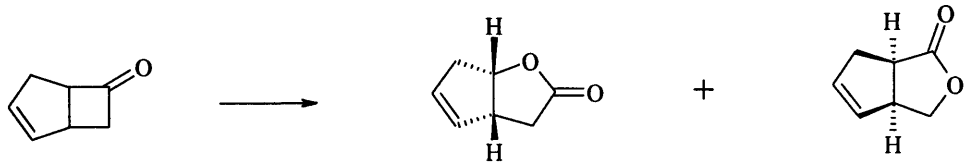


Figure 1.31: Regiodivergent bio-oxidation of racemic bicyclic ketones where X= carbon

One such example of regiodivergent reaction is that of the CHMO enzyme isolated from *Acinetobacter calcoaceticus.* This enzyme system has been extensively studied (Alphand *et al.*, 1990a/b,1992; Roberts *et al.*, 1993 and Taschner *et al.*, 1993) due to the enantiomerically pure lactones formed. It was first cloned and over-expressed by Chen and co-workers in the late eighties (Chen *et al.*, 1988). However, it was found that a lactone hydrolase was present which depending on the substrate used posed a problem for product yield. Recently, the exact gene sequence of this CHMO has been isolated and cloned in to *Escherichia coli* in an L-arabinose inducible vector (TOP10 pQR239), (Doig *et al.*, 2001). This CHMO has the ability to carry out Baeyer-Villiger oxidation and resolve the racemic bicyclo ketone (+/-) bicyclo[3.2.0]hept-2-en-6-one to yield two lactones, the expected "normal" lactone (-)-(1*S*,5*R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one and the "abnormal" (-)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one respectively in a 1:1 ratio (Doig *et al.*, 2002) figure 1.32.



(+/-)bicyclo[3.2.0]hept-2-en-3-one

2-oxabicyclo[3.3.0]oct-6-en-3-one
(-)-(1*S*,5*R*)

3-oxabicyclo[3.3.0]oct-6-en-2-one
(-)-(1*R*,5*S*)

Figure 1.32: Conversion of (+/-) bicyclo[3.2.0]hept-2-en-6-one to (-)-(1*S*,5*R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one and (-)-(1*R*,5S)-3-oxabicyclo[3.3.0]oct-6-en-2-one respectively in a 1:1 ratio by the over-expressed CHMO (*E. coli* TOP10 pQR239)

The use of the AcCHMO for this reaction provides two regioisomeric lactones with high enantioselectivity. However, obtaining regio-pure lactones is of high interest and screening for organisms that provide a regioselective reaction was performed by Alphand and co workers. It was found that the bioconversion of (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone by the fungus *Cunninghamella echinulata* NRRL 3655 resulted in the regioselective production of "abnormal" 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone with high enantioselectivity (Alphand *et al*, 2000). This regioselective property is highly attractive. Firstly, as the production of 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone by classical chemical synthesis is extremely difficult and secondly, the use of chromatographic separation of the lactone regioisomers produced via the AcCHMO reaction is avoided.

1.4 Thesis Aims and Objectives

1.4.1 Project Aims

This project will examine the usefulness of the Baeyer-Villiger monooxygenase in the fungus *Cunninghamella echinulata* NRRL 3655. It has been shown to carry out the regioselective bioconversion of racemic (+/-) bicyclo[3.2.0]hept-2-en-6-one substrate where (-)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one is predominantly formed (20:1 ratio 3-oxa lactone : 2-oxa lactone) during the reaction (Alphand *et al.*, 2000). It is this difference in ratio that is highly appealing for two reasons; Firstly, classical chemical synthesis of the lactones primarily results in the formation of the 2-oxabicyclo[3.3.0]oct-6-en-3-one lactone, or a mixture of both lactones which involves a time consuming and hazardous separation technique. Secondly, as a complementary system to the AcCHMO where both lactone regioisomers are produced via a regiodivergent reaction. Access to the pure 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone provides the starting point for further synthesis of biologically active compounds such as multifidene and Baclofen® (discussed further in chapter 2).

It is this difference in the lactone regioselectivity that makes this fungal system novel compared to the existing AcCHMO system. Using the BVMO from *C. echinulata* has the advantage over AcCHMO as the use of a racemic ketone substrate to yield regio and enantio pure lactone is possible, table 1.6. However, this system has disadvantages as highlighted in table 1.6 and it is the aim of this project to address these issues and be able to map out a path forward for scalable exploitation.

This will involve the isolation and purification of the CeBVMO protein leading to crystallisation and structure elucidation. Cloning and over-expression of the CeBVMO in to an alternate host will enable the key features of this enzyme to be addressed.

Table 1.6: Comparison of AcCHMO with the CeBVMO

	Reaction	Advantages	Disadvantages
CHMO + racemic ketone	Non regioselective and enantiodivergent	Cloned and over-expressed in *E. coli.* Well studied system with regards to growth characteristics, enzyme kinetics and stability	Both lactone regioisomers produced chromatographic separation necessary
CHMO + enantiopure ketone	Regioselective	Cloned and over-expressed in *E. coli.* Well studied system with regards to growth characteristics, enzyme kinetics and stability	Chemical separation and purification of ketones necessary-difficult and time consuming process
BVMO + racemic ketone	Regioselective and enantioselective	Resolution of racemic ketone in to enantio and regio pure lactones	*C. echinulata* fungal system not fully characterised Long growth time necessary (+8 days) Low expression of BVMO in fungus Chromatographic separation lactone and enriched ketone necessary

1.4.2 Conceptual Challenges

Little is known about the growth of the fungus therefore growth characteristics will be investigated to enable effective growth on a large scale in order to obtain maximum CeBVMO enzyme yields for extraction and purification.

In comparison to the AcCHMO isolated from *A. calcoaceticus*, (which is a class II pathogen making it undesirable to use at a commercial scale), *C. echinulata* itself is undesirable as a host organism due to the long growth time required for CeBVMO synthesis. It is therefore necessary to isolate the CeBVMO and clone in to an alternate host reducing growth time for CeBVMO synthesis. Obtaining the CeBVMO N-terminal sequence data will enable oligonucleotide primers to be designed to target and isolate the CeBVMO DNA sequence which will allow the gene to be cloned and over-expressed in an alternate host organism, such as *E. coli.* Characterisation studies will allow the key features of this enzyme to be addressed, i.e. substrate specificity and regio and enantioselectivities and how they differ from the established AcCHMO.

Isolation of the CeBVMO will allow crystal structure data to be obtained allowing the understanding of the relationship between the structure and functions of this enzyme to be investigated.

Investigating *C. echinulata's* ability to perform multistep bioconversions using multiple pathway enzymes. Using a multi enzyme pathway system will allow the use of chemically different starting substrates to yield the desired products along with the synthesis of regio and enantio pure intermediates.

Ultimately, the end goal of this thesis is to gain a further understanding of the CeBVMO present in the fungus *C. echinulata* and clone into an alternate host organism. In turn, this will allow the development of a continuous process where substrate feeding and product recovery are possible resulting in regio and enantio pure lactone production.

CHAPTER 2

Introduction to *Cunninghamella echinulata*

2.1 Introduction

In this chapter the growth of *C. echinulata* during submerged fermentation and plate culture will be investigated. Understanding the key growth requirements and growth cycle will allow successful production of the fungal mass on a large scale which is essential due to the low expression of this enzyme. This will allow the isolation and extraction of the BVMO from *C. echinulata.*

Substrate specificity of the BVMO will be investigated using a range of cyclic ketone substrates which will be compared to that of the AcCHMO isolated from *A. calcoaceticus.*

2.2 Cunninghamella sp

2.2.1 Introduction

The fungus *Cunninghamella* sp. is a filamentous zygomycete consisting of over 120 strains of the genus. A recent study in to the phylogeny of zygomycetes placed *Cunninghamella* in to Cunninghamellaceae, zygomycota, order Mucorales. It is identified to belong to Mucorales on the basis of the presence of a columella. This is a bulbous vesicle structure which occurs at the base of the asexual sporangium (Voigt and Wöstemeyer, 2001; figure 2.1) The strains can be classified in to 8 taxa belonging to 4 species of *Cunninghamella,* these are *C.blakesleena, C.echinulata, C.elegans* and *C.polymorpha.*

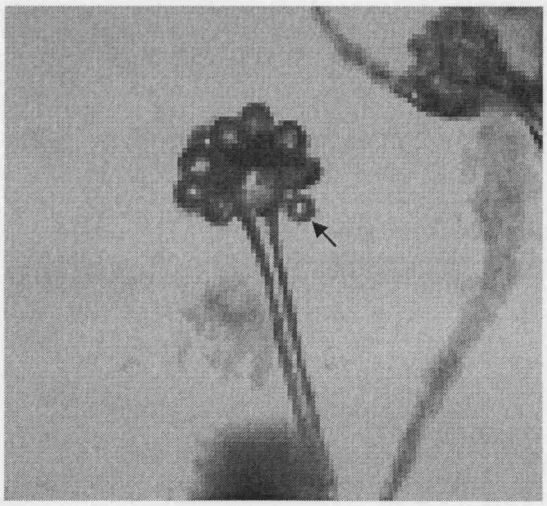


Figure 2.1: C. *echinulata* showing broad aseptate hyphae with long branched sporangiophores ending in swollen vesicles covered in denticles. Attached to the denticles are round oval sporangiolum (arrowed)

All but one species of *Cunninghamella* is non-pathogenic, *C. blakesleena.* This has been shown to cause human disease as an opportunistic organism affecting immunocompromised patients. To date 30 cases of infection caused by *C. blakesleena* are documented (Garey *et al.*, 2001). The mortality rate is high, with the lungs being the most commonly targeted organ causing fatal cases of fungal pneumonia.

2.2.2 Fungal Properties

Cunninghamella has shown the ability to metabolise various xenobiotics in ways similar to those found in mammalian systems. An earlier study using *C. elegans* ATCC 36112 was performed to provide enzymatic data and mechanisms using various metabolites (Zhang *et al.*, 1996). The data obtained showed that the fungus contained high activity of glutathione S-transferase (GST), which catalyses the transfer of the tripeptide glutathione to a co-substrate containing a reactive centre to form a S-glutathionylated reaction product, and an aryl PAPS sulfotransferase, which transfer a sulfuryl group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to the hydroxyl/amino groups of acceptor molecules. Both of these enzymes were shown to act on 1-naphthol and 1-chloro-2,6-dinitrobenzene, figure 2.2.

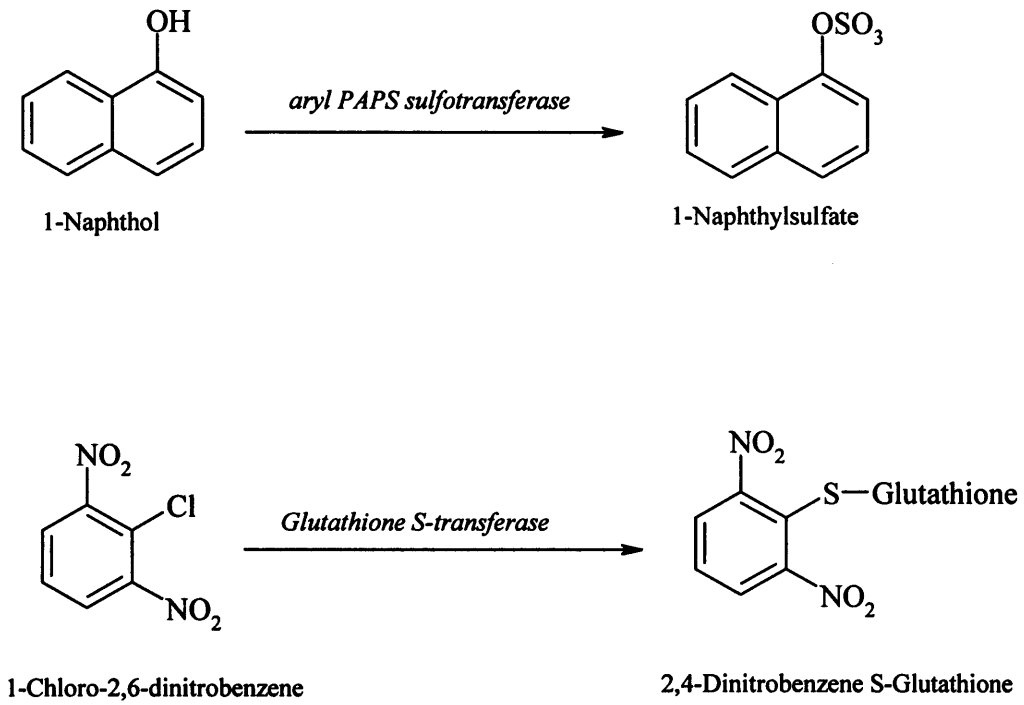


Figure 2.2: Enzymatic reactions using 1-naphthol and 1-chloro-2,4-dinitrobenzene as substrates using *C. elegans*

Past studies have identified the cDNA encoding 6-phosphoglycerate dehydrogenase which is a major enzyme in the pentose phosphate pathway responsible for the conversion of 6-phosphogluconic acid to ribulose-5-phosphate and CO_2 (Wang *et al.*, 1998) along with the gene encoding enolase which catalyses the conversion of glycerate-2-phosphate to phosphoenol pyruvate in *C. elegans* (Wang, 2000).

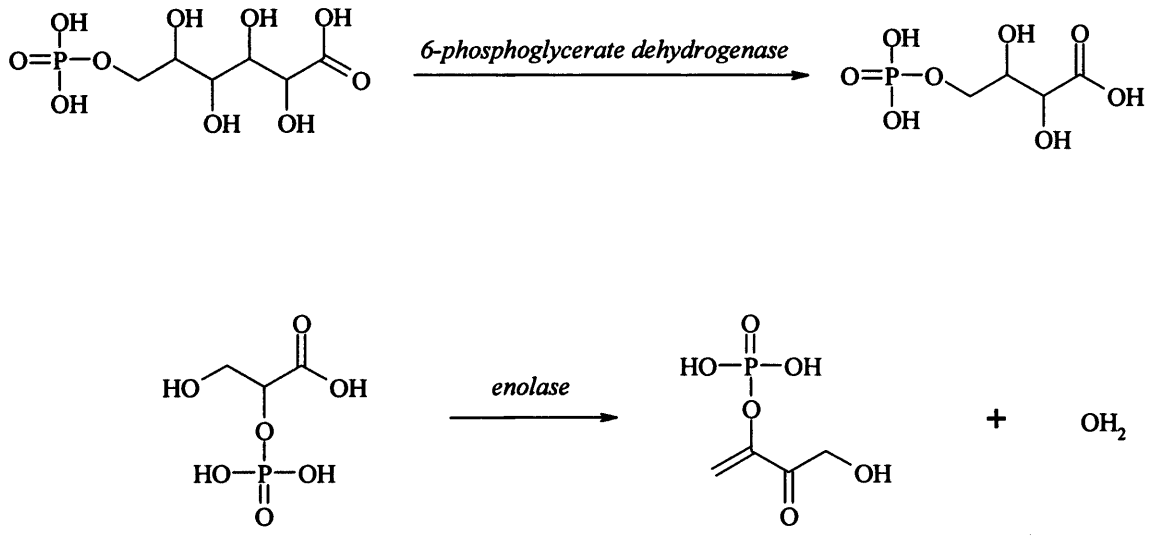


Figure 2.3: Conversion of 6-phosphogluconic acid to ribulose-5-phosphate by 6-phosphoglycerate dehydrogenase and the conversion of glycerate-2-phosphate to phosphenol pyruvate by enolase

The 6-phosphoglycerate dehydrogenase gene has been shown to contain 1458 bases encoding a protein containing 485 amino acids with a mass of 53 kDa, and related to yeast 6-phosphoglycerate dehydrogenase within the phylogenetic tree.

The gene encoding enolase has a mass of 46 kDa and shows homology with the enolase of other fungi, including yeast. Overexpression of the genes in to *E. coli* has been successful (Wang *et al.*, 2000).

Nuclease C1 (Michels *et al.*, 1998) and cytochrome P450 oxidoreductase (Yadav and Loper, 2000) have also been identified in *C. elegans* and *C. echinulata.*

Nucleases act on DNA and RNA to release 5' or 3' phosphate products. Several fungal extracellular nucleases are known, S1 in *Aspergillus oryzae* and P1 in *Penicillium citrinum*, both of which are well characterized (Iwamatsu *et al.*, 1991; Sück *et al.*,

1993). Experiments have shown that *C. echinulata* contains three distinct extracellular nucleases. The major nuclease, termed C1 is 30 kDa composed of 252 residues. Homology studies indicate similarities with mitochondrial nucleases found in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*.

Since the early 1970s cytochrome P450s have been known to be present in the fungal enzyme system for xenobiotic biotransformation (Ferris *et al.*, 1976). The gene isolated in *C. elegans* was shown to contain 2420 base pairs encoding 710 amino acids whereas a partial sequence from *C. echinulata* contained 2074 base pairs was isolated. Phylogenetic comparison of the P450 gene from *C. elegans* indicated a close relationship to animal P450s compared to that of yeast P450.

2.2.3 Bioconversions involving *C. echinulata*

In 1997 it was reported by Mazzini and co workers (Mazzini *et al.*, 1997), that *C. echinulata* NRRL 3655 was able to perform an enantioselective synthesis step in the production of Baclofen®. The step involves the novel oxidation of 3-(4'-chlorophenyl)-cyclobutanone to produce (*R*)-(-)-chlorophenyl lactone, a valuable starting point for the synthesis of Baclofen® ,which is an agonist of γ-aminobutyric acid, figure 2.4. Baclofen® reduces glutamate release in the spinal cord producing an antispastic effect, which is useful in controlling diseases such as multiple sclerosis.

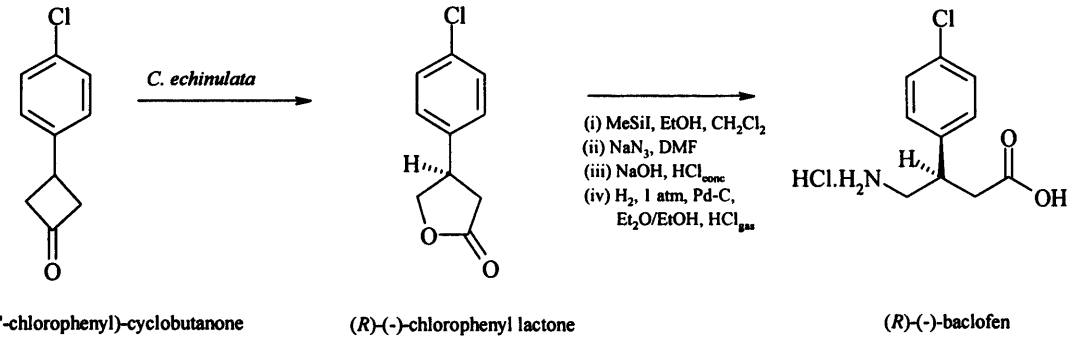


Figure 2.4: The initial BVMO catalysed step in the synthesis of Baclofen®

The pathogenic *C. blakesleena* has been reported to be useful for the hydrolysis of a lactone to yield an antidepressant drug, Milnacipran® (Viazzo *et al.*, 1996). Milnacipran® possesses similar properties to that of fluoxetine (Prozac®) however upon hydrolysis of the key lactone two enantiomers are produced. Each enantiomer has different pharmacokinetic properties but it is the (1*S*,2*R*) enantiomer that shows activity. Therefore an efficient procedure was required to selectively produce the active enantiomer. Using *C. blakesleena* a 99% ee of the (1*S*,2*R*) enantiomer was obtained, figure 2.5. However, the use of this fungus in scale up and commercialisation is problematic due to its pathogenicity.

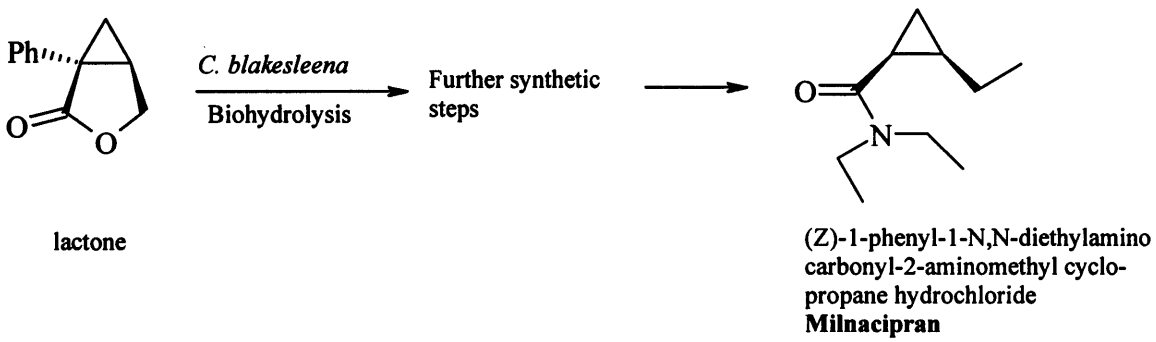


Figure 2.5: Scheme showing position of *C. blakesleena* in production of Milnacipran®

Lebreton and co workers showed the novel use of *C. echinulata* to perform a Baeyer-Villiger oxidation reaction as part of a five step synthesis of brown algae pheromones, (+)-Multifidene and (+)-Viridiene (Lebreton *et al.*, 1996). Figure 2.6 outlines the steps involved in pheromone production using (+/-) bicyclo[3.2.0]hept-2-en-6-one as a starting material. *C. echinulata* actively performs a Baeyer-Villiger oxidation converting the ketone into 3-oxabicyclo[3.3.0]oct-6-en-3-one which then undergoes further chemical synthesis to yield the pheromones.

The pheromones are secreted to stimulate and attract male species in order to initiate sexual fusion. Viridiene and Multifidene are found in *Chorda tomentosa* and *Cutleria multifida* respectively (Maier *et al.*, 1984).

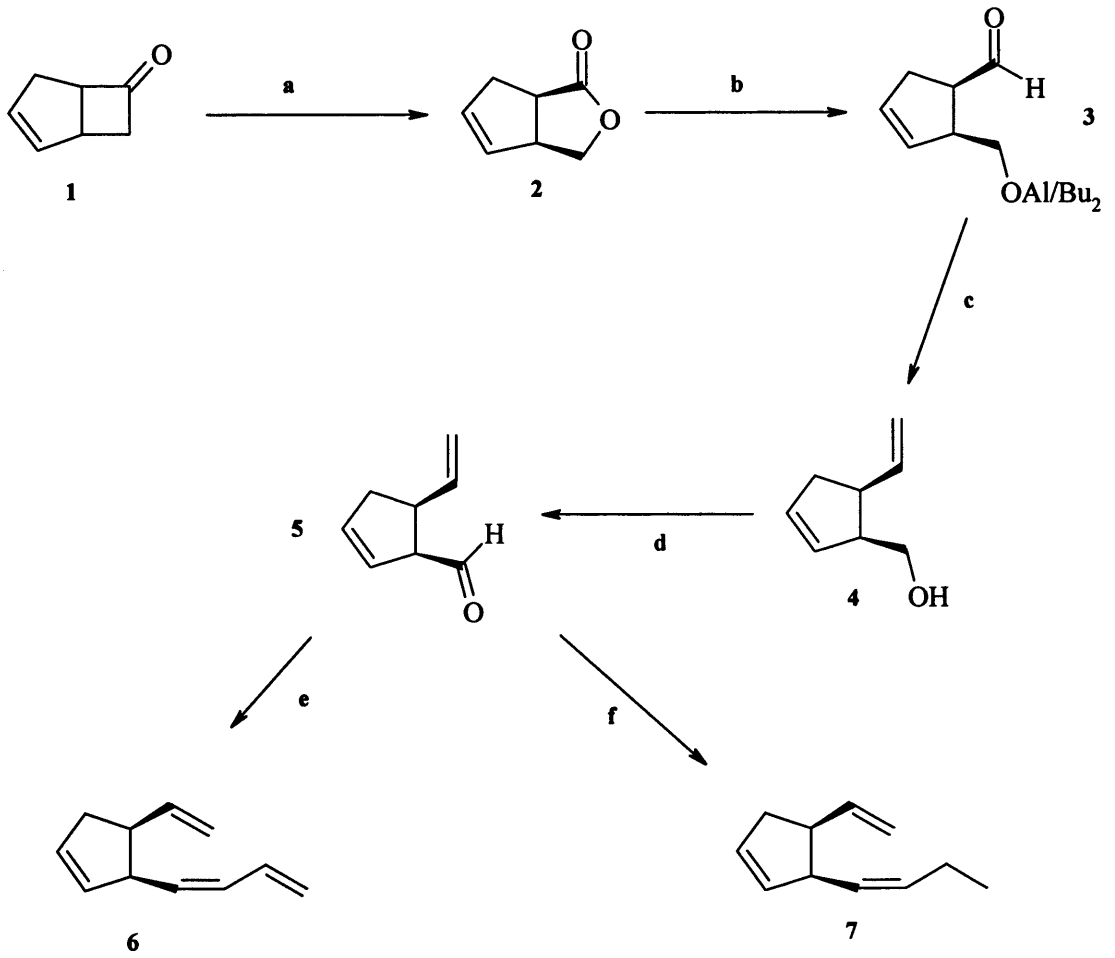
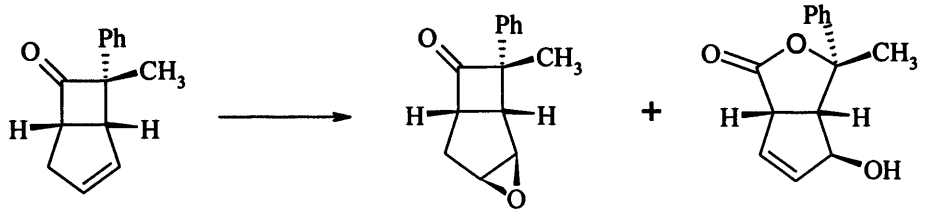


Figure 2.6: Conversion of Bicyclo[3.2.0]hept-2-en-6-one (3) via enzymatic Baeyer-Villiger oxidation to yield Viridiene (6) and Multifidene (7)

(1)- (+/-) bicyclo[3.2.0]hept-2-en-6-one; (2)- 3-oxabicyclo[3.3.0]oct-6-en-3-one;
(3)- 2-Ethyl-cyclopent-3-enecarbaldehyde; (4)- Hydroxyolefin;
(5)- 5-Vinyl-cyclopent-2-enecarbaldehyde; (6)- Viridiene; (7)- Multifidene
(a)- Cunninghamella echinulata; (b)- DIBAL-H, toluene, -78°C, 1h; (c)- $Ph_3P{=}CH_2$, THF, -78°C; (d)- $(ClCO)_2$ DMSO, THF, -78°C, 3h then Et_3N, -78°C to 0°, 1h; (e)- $Ph_3P{=}CH\text{-}CH{=}CH_2$, -100°C to rt, 12h then 4-phenyl-2,3,4-triazoline-3,5-dione, THF, rt, 5 min; (f)- $Ph_3P{=}CH\text{-}Et$, -78°C to rt, 12h.

Fairlamb and co-workers have successfully used *C. echinulata* to perform a Beyer-Villiger oxidation and allylic oxidation of 7-*exo*-methyl-*7-endo*-phenylbicyclo[3.2.0]hept-2-en-6-one to yield an hydroxylactone allowing access to novel chiral ligands (Fairlamb *et al.*, 2004), figure 2.7



7-*exo*-methyl-7-*endo*-phenylbicyclo[3.2.0]hept-2-en-6-one

epoxide

hydroxylactone

Figure 2.7: Beyer-Villiger oxidation and allylic oxidation of 7-*exo*-methyl-*7-endo*-phenylbicyclo[3.2.0]hept-2-en-6-one to yield an epoxide and hydroxylactone

Interestingly, both oxidation steps are performed regio and stereoselectively which provides access to valuable three chiral centred chemical precursors.

2.2.4 Using *Cunninghamella* for modelling mammalian metabolism

The interest in the use of *Cunninghamella* to mimic mammalian systems has increased over the years, primarily in the drug industry. Over the past decade several species from the *Cunninghamella* family have been used to model mammalian systems. An example of this is the bioconversion of *RAC*-Mexiletine. This
is an antiarrhythmic drug used to treat ventricular arrhythmias. In 1996 Freitag and co workers used *C. echinulata* to metabolise *RAC*-Mexiletine in to two metabolites, *p*-hydroxymexiletine and hydroxymethylmexiletine, both observed in human metabolism (Freitag *et al.*, 1997) figure 2.8.

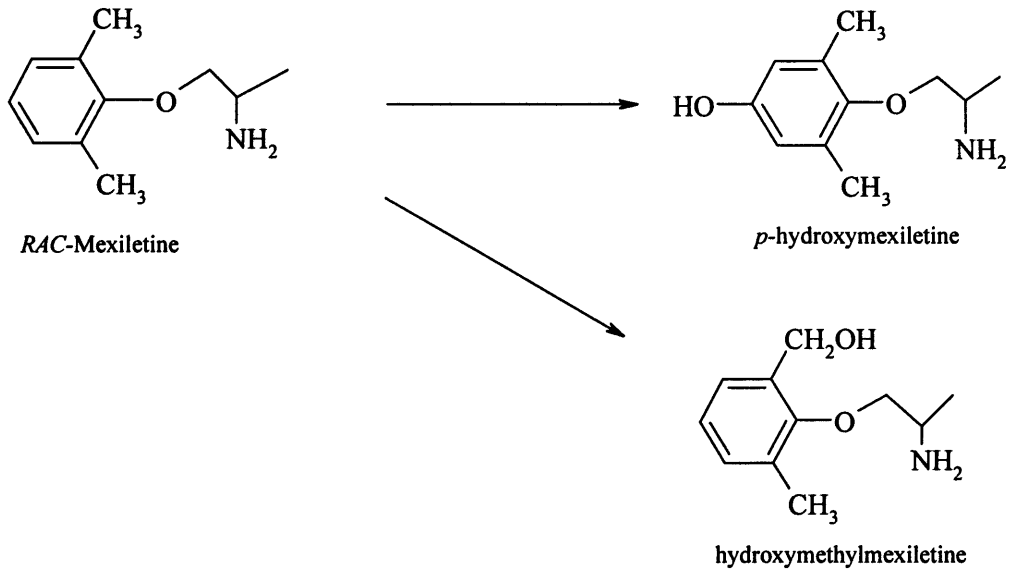
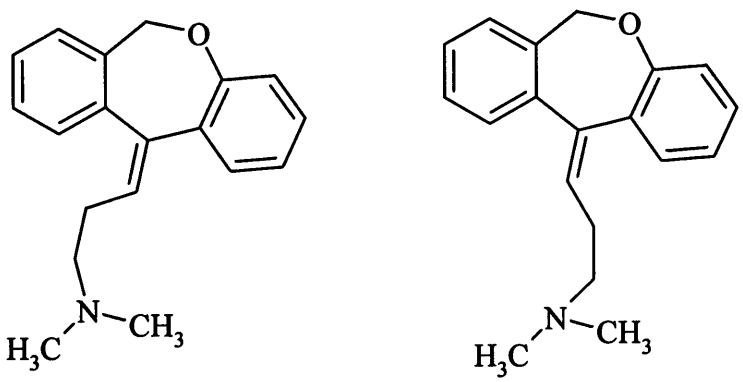


Figure 2.8: Metabolism of *RAC*-Mexiletine to *p*-hydroxymexiletine and hydroxymethylmexiletine

In 1999 Moody and co workers (Moody *et al.*, 1999) performed bioconversion with doxepin using *Cunninghamella elegans*. Doxepin is an antidepressant drug having similar structures to amitriptyline and imipramine. It is made up of two isomers, *trans* (E) and *cis* (Z) forms with the *cis* form showing the highest pharmacological effect, figure 2.9. Using *C. elegans* doxepin was metabolized to 16 metabolites, six of which were the same as the human metabolites, and nine new metabolites were identified. These new metabolites will determine whether there is a stereoselective metabolism of the doxepin isomers.



trans-(E) *cis*- (Z)

Figure 2.9: Structures of Doxepin *trans* (E) and *cis* (Z)

C. echinulata has demonstrated its unique property to metabolise xenobiotics in a way similar to mammalian metabolism making it a valuable candidate as a model for mammalian drug metabolism. Several enzymes have been identified and isolated from *Cunninghamella* which provides valuable information on the use of the enzymes for biotransformation of xenobiotics or other compounds.

2.2.5 Baeyer-Villiger oxidation reaction using *C. echinulata*

As described briefly in section 1.3.3, *C. echinulata* has been shown to contain a BVMO reported by Alphand and co workers (Alphand *et al.*, 2000). However, unlike the AcCHMO where both (-)-(1*S*,5*R*)-2-oxabicyclo[3.30]oct-6-en-3-one and (-)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one lactone products are formed in a 1:1 ratio the CeBVMO reaction performs an regioselective reaction, predominantly selective for the (-)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one lactone in a 1:20 ratio ((-)-2-ox: (-)-3-ox), figure 2.10.

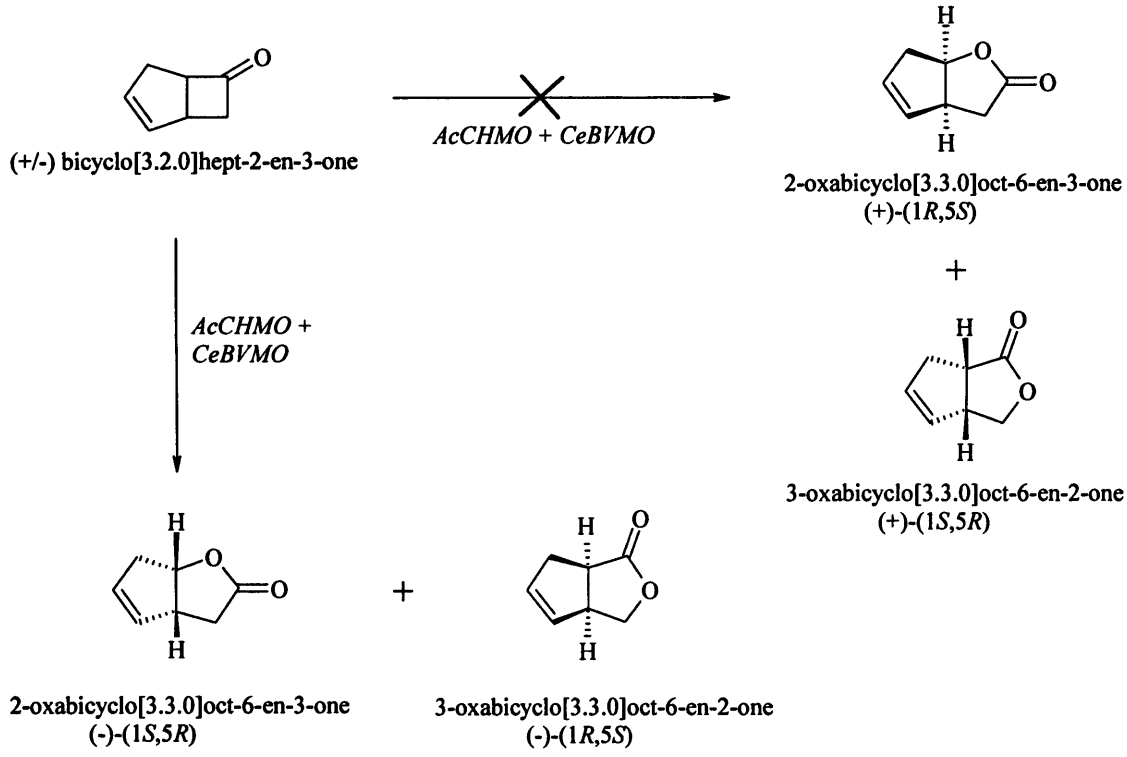


Figure 2.10: Specificity of AcCHMO and CeBVMO using racemic ketone substrate to form both (-)-(1*S*,5*R*)-2-oxabicyclo[3.30]oct-6-en-3-one and (-)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one lactone products X :- no conversion

This biochemical oxidation reaction produces two enantiopure lactones that provide the starting precursors for the production of pharmaceuticals and commercially important chemicals such as prostaglandins (Newton *et al.*, 1980).

2.3 Materials and Methods

Unless stated otherwise all chemicals were of the highest purity available from Sigma-Aldrich Chemical Company (Poole, Dorset, U.K.). Growth media components were obtained from Oxoid Ltd (Basingstoke, Hants, U.K.). *Cunninghamella echinulata* NRRL 3655 was obtained from LGC (Teddington, Middlesex, U.K.).

2.3.1 Growth of *C. echinulata*

2.3.1.1 Plate cultivation on solid media

The fungus was cultivated on thinly poured potato dextrose agar plates at 30°C and growth was observed on a daily basis for a total of seven days. Images were captured using a manual image analysis system consisting of a microscope (Leica DMRA2), a CCD camera with captured images of 760 × 574 pixels and a PC with framegrabber and image analysis software (Leica Qwin, Leica Microsystems UK Ltd, Milton Keynes, Beds, U.K.).

2.3.1.2 Fermentations

Fermentations were performed using corn steep liquor media: corn steep liquor (20 g/L), glucose (4 g/L), KH_2PO_4 (1 g/L), K_2HPO_4 (2 g/L), $NaNO_3$ (2 g/L), KCl (0.5 g/L), $MgSO_4$ (0.5 g/L), $FeSO_4$ (0.02 g/L), PEG antifoam (0.5 ml/L). Potato dextrose agar was used to cultivate the fungus on slopes and petri dishes (pH 5.5).

2.3.1.3 Inoculum

100 ml corn steep liquor broth in a 250 ml flask was inoculated with spores taken from a corn steep liquor media slope and incubated at 30°C for 72 hours as a static culture. Subsequently this culture was shaken vigorously for one minute resulting in spore release from the biomass. 50 ml of this culture was used to inoculate 1.5 L corn steep liquor submerged fermentation, which was carried out for five days, or until the pH reached 8.5, after which the cells were harvested.

2.3.1.4 1.5L Fermentation

Fermentations were carried out in a LH 210 series 2 L stirred tank fermenter (working volume 1.5 L) (Bioprocess Engineering Services, Charing, Kent, U.K), equipped with 2 six-blade Rushton disc turbines, with the baffles removed. The aeration rate was kept at 3 vvm (5 L min) via a submerged sparger, agitation speed at 450 rpm and a constant temperature of 30°C. DOT was measured by a polarographic oxygen electrode (Ingold-Mettler Toledo, Greifensee, Switzerland).

2.3.2 Pellet preparation for scanning electron microscope (SEM)

The fungal pellets from the fermentation were harvested by filtration though a Miracloth membrane (20-22 μm pore size) (Calbiochem, Beeston, Notts, U.K.) and washed with 300 ml of 100 mM sodium phosphate buffer (pH 8). Several pellets were selected and preserved with 10 ml of SEM preservation buffer (sodium phosphate buffer (0.1 M, pH 7.2), 3% glutaraldehyde) for 24 hours, fixed (1% osmium tetroxide) overnight, dehydrated (increasing percentage of acetone stepwise from 10% to 95%) followed by critical point drying and mounting. Images were viewed using a Cambridge S100 SEM (LEO Electron Microscopy Ltd, Cambridge, U.K.)

2.3.3 BVMO activity assay

In a typical procedure the fungal pellet mass was harvested by filtration through a Miracloth membrane and washed with 300 ml of 100 mM sodium phosphate buffer (pH 8). 5 g of cells were re-suspended into a 250 ml non-baffled shake flask containing 100 ml of 100 mM sodium phosphate buffer (pH 8) and incubated with 100 μl (+/-) bicyclo[3.2.0]hept-2-en-6-one in a reciprocating shaker at 170 rpm, 28°C for 24 hours.

2.3.4 Analytical Gas Chromatography (G.C.)

All metabolites were identified by comparison of their retention times using an XL-2 gas chromatograph with flame ionisation detector (Perkin-Elmer, Norwalk, CT, USA) fitted with a ZB1 non-polar dimethylsiloxane column (30 m × 0.25 mm × 0.25 μm) (Phenomenex, Macclesfield, Cheshire, U.K.) with helium as the mobile phase with those of authentic compounds synthesised by classical chemical reactions or commercially available. 400 μl supernatant from the biotransformation was extracted with an equal volume of ethyl acetate containing naphthalene as the internal standard. The G.C. operating temperatures were set as follows: column 110°C, injector 250°C and detector 250°C. Retention times for bicyclo[3.2.0]hept-2-en-6-one, 3-oxabicyclo[3.3.0]oct-6-en-2-one, 2-oxbicyclo[3.3.0]oct-6-en-3-one and naphthalene were 1.8 mins, 3.4 mins, 3.5, mins and 4.0 mins, respectively.

2.3.5 Nitrate Assay

Solutions were prepared as follows:

Solution A: Salicylic acid (5% w/v) in concentrated sulphuric acid
Solution B: NaOH (2M)
Standard solution: $NaNO_3$ (0.84 g/L,10 mM)

A 20 μl sample of fermentation broth was added to 80 μl solution A, mixed thoroughly and incubated at room temperature for 20 mins after which 1.9 ml solution B was added and cooled to room temperature. Optical density was measured at 410 nm (ATI Uvicam UV/Vis Spectrophotometer, Uvicam Ltd, Cambs, UK).

2.3.6 Ammonia Assay

Solutions were prepared as follows:

Solution A: Phenol (10 g) and sodium nitrosopentacyanoferrate (III) (5 mg) was added to water (1L) and mixed thoroughly.
Solution B: NaOH (5 g) and sodium hypochlorite solution (8.4 ml) was added to water (1L) and mixed thoroughly.
Standard solution: NH_4Cl (0.16 g, 2.99 mM) was made up to 1L using water

A 20 µl sample of fermentation broth was added to solution A (1 ml) and mixed. Solution B (1 ml) was added and mixed thoroughly followed by incubation at 37°C. After 15 mins the optical density was read at 625 nm (ATI Uvicam UV/Vis Spectrophotometer, Uvicam Ltd, Cambs, UK).

2.3.7 Substrate Specificity

2.3.7.1 Inoculum

Two flasks (2L) containing 500 ml corn steep liquor broth were inoculated with spores taken from a corn steep liquor media slope and incubated at 30°C for 72 hours as a static culture. Subsequently this culture was shaken vigorously for one minute resulting in spore release from the biomass. The contents of both flasks was used to inoculate 15 L corn steep liquor submerged fermentation, which was carried out for five days, or until the pH reached 8.5, after which the cells were harvested.

2.3.7.2 15L submerged fermentation

Fermentations were carried out in a LH 1075 series 20 L stirred tank fermenter (working volume 15 L) (Bioprocess Engineering Services, Charing, Kent, U.K), equipped with 3 six-blade Rushton disc turbines, with the baffles removed. The aeration rate was kept at 3 vvm (5 L min) via a submerged sparger, agitation speed at 450 rpm and a constant temperature of 30°C. DOT was measured by a polarographic oxygen electrode (Ingold Messtechnik, Urdorf, Switzerland) and pH measured by a steam sterilisable Ingold pH probe (Ingold-Mettler Toledo, Greifensee, Switzerland).

The inlet and exhaust gases were filtered through 0.2 μm filters (Gelman Sciences, Ann Arbor, MI, USA) and the composition of the exhaust gas was determined using mass spectrometry (Prima 600, VG Gas Analysis, Winsford, Cheshire, UK) Data logging and exhaust gas measurements were recorded with the TS-DAS program (real-time data acquisition system) (Acquisition Systems, Guildford, Surrey, UK).

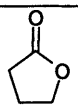
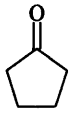
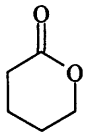
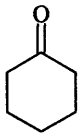
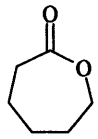
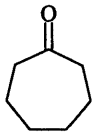
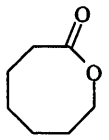
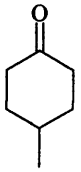
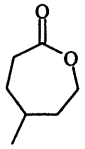
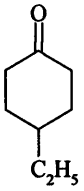
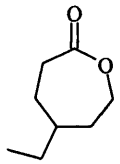
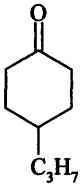
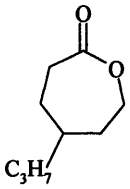
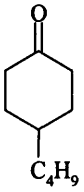
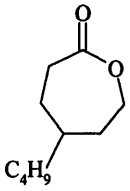
2.3.8 Activity and substrate inhibition studies using bicyclo[3.2.0]hept-2-en-6-one

The fungal pellets from the fermentation were harvested by filtration through a Miracloth membrane (20-22 μm pore size) and washed with 1.5L 100mM sodium phosphate buffer (pH 8) to remove residual fermentation media. Non-baffled shake flasks (250 ml) were set up containing 5 g of pellets (wet weight), which were suspended in 50 ml 100 mM sodium phosphate buffer (pH 8). (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone was added at concentrations 5,10,20,30,40,50 and 100 mM and incubated in a reciprocating shaker at 170 rpm, 28°C for 4 days.

2.3.9 Activity screening and inhibition studies using cyclic ketones

The fungal pellets from the fermentation were harvested by filtration through a Miracloth membrane (20-22 μm pore size) and washed with 1.5L 100mM sodium phosphate buffer (pH 8) to remove residual fermentation media. Non-baffled shake flasks (250 ml) were set up containing 5 g of pellets (wet weight), which were suspended in 50 ml 100 mM sodium phosphate buffer (pH 8). Ketones (table 2.1) were added at concentrations 5,10,20,30,40,50 and 100 mM and incubated in a reciprocating shaker at 170 rpm, 28°C for 4 days.

Table 2.1:Ketones and their corresponding lactones.

Ketone		Lactone	
Cyclobutanone		Dihydro-furan-2-one	
Cyclopentanone		Tetrahydro-pyran-2-one	
Cyclohexanone		Oxepan-2-one	
Cycloheptanone		Oxocan-2-one	
4-methylcyclohexanone		5-methyl-oxepan-2-one	
4-ethylcyclohexanone		5-ethyl-oxepan-2-one	
4-propylcyclohexanone		5-propyl-oxepan-2-one	
4-butylcyclohexanone		5-butyl-oxepan-2-one	

2.3.10 Synthesis of cyclic lactones

2.3.10.1 Synthesis of Dihydro-furan-2-one and Tetrahydro-pyran-2-one

Dihydro-furan-2-one and tetrahdro-pyran-2-one were synthesis following procedures according to Lai and co-workers (Lai *et al.*, 2002).

Tetrahydrofuran (860 µl) was added to a round bottomed flask containing acetone (10ml), potassium permanganate (1.6 g, 1.0 mmol) and iron (III) chloride (1.0g, 6.2mmol). The mixture was cooled to –78°C and stirred. After two hours the cooling bath was removed and allowed to warm to room temperature and stirred for a further 16 hours. Dichloromethane (20 ml) was added to the reaction mixture and filtered, decolourised over charcoal, dried over $MgSO_4$ and vacuum dried to yield a colourless oily product. Tetrahydro-pyran-2-one was synthesised as above starting from tetrahydropyran. Spectroscopic properties (^{1}H NMR) of the above synthesised compounds were consistent with their assigned structure as reported in literature (Lai *et al.*, 2002).

2.3.10.2 Synthesis of oxocan-2-one

Oxocan-2-one was synthesised following procedures according to Bidd and co workers (Bidd *et al.*, 1983).

Dichloromethane (6.4 ml) was added to acetic anhydride (5 ml) in a cooled reaction flask fitted with a condenser. Hydrogen peroxide (30%, 4 ml) was added drop wise over 30 minutes after which maleic anhydride (4 g) was added and stirred with cooling. After one hour cooling was removed and the reaction allowed to heat up to reflux. After no more spontaneous reflux was visible (approx 2 hours) the reaction mixture was left to cool to room temperature (approx 2 hours). Cycloheptanone (1 ml) was added and gentle reflux was maintained for 15 hours. The mixture was cooled and filtered to remove the maleic acid. The filtrate was washed with water (3 washes), 10% potassium hydroxide and 10% sodium sulfite (3 washes) followed by water (3 washes). The resulting mixture was dried over $MgSO_4$ and evaporated to yield the lactone. Spectroscopic properties (^{1}H NMR) of the above synthesised compounds were consistent with their assigned structure as reported in literature (Bidd *et al.*, 1983).

2.3.10.3 Synthesis of di-substituted oxepan-2-ones

mCPBA (2.07 g, 12 mmol/L) and $NaHCO_3$ (1.008 g, 12 mmol/L) were added to dry dichloromethane (50 ml) and stirred under argon for one hour. 500 µl ketones (4-methylcyclohexanone, 4-ethylcyclohexanone, 4-propylcyclohexanone and 4-butylcyclohexanone) were added and stirred for 16 hours at room temperature. Once complete the reaction was neutralised with 10% $NaCO_3$ (3 washes), brine (3 washes) ,dried over $MgSO_4$ and concentrated under reduced pressure to yield the final lactone products. (Jagt *et al.*, 2001)

The spectroscopic properties (^1H NMR) of all the above synthesised compounds were consistent with their assigned structure according to literature (Jagt *et al.*, 2001).

2.4 Results

2.4.1 Fungal growth

Fungi that belong to the Zygomycota phyla grow rapidly producing many sporangiospores. In previous work it has been found that those classified under the order Mucorales often grow best at pH 6 (Levetin and Caroselli, 1976).

In this thesis, studies on the growth of *C. echinulata* have been carried out and the data recorded pictographically as detailed in figure 2.11. It has been found that mycelium bear from spores within 4 hours and formation of long hyphae is rapid (figure 2.11 : A and B). Internal fluid can be seen moving throughout the coencytic mycelium towards the growing mycelium tip.

At points along the mycelium, tubular denticles bud forming new, short lengths of mycelia (sporangiophores) (figure 2.11: C), which bear sporangium at the tip. Electron microscope studies by Khan showed that the sporangium contains nuclei, free ribosomes, mitochondria, rough endoplasmic reticulum and vacuoles (Khan and Talbot 1975). It can be seen that it is made up of a two layered wall consisting of a thin outer wall and a thicker inner wall (figure 2.11: D). Internal pressure builds up within the sporangium causing small denticles to be formed at localised weak spots on the wall. Sporangia develop at the ends of these denticles which contain similar cytoplasmic contents to that of the sporangium, however, the sporangia wall consists of one layer only. As the sporangia matures a single sporangiospore could be seen forming inside the sporangia and the thickening of the cell wall along with spines developing on the outer surface. These spines are hollow calcium oxalate crystals and electron microscope studies by Hawker and co workers showed them as having a circular base which are raised on the upper surface giving them a dome like shape. The spine then develops out from this raised centre point and grows in a tapered barrel shape developing a sharp tip at the end (Hawker *et al.*, 1970).

After five days growth, fully matured sporangia were observed containing sporagiospores which become separated from the sporangium (figure 2.11: E). These sporangiospores are the fruiting bodies of the fungus formed by asexual reproduction and are naturally dispersed passively through contact with water forming spore droplets or dispersed by air currents

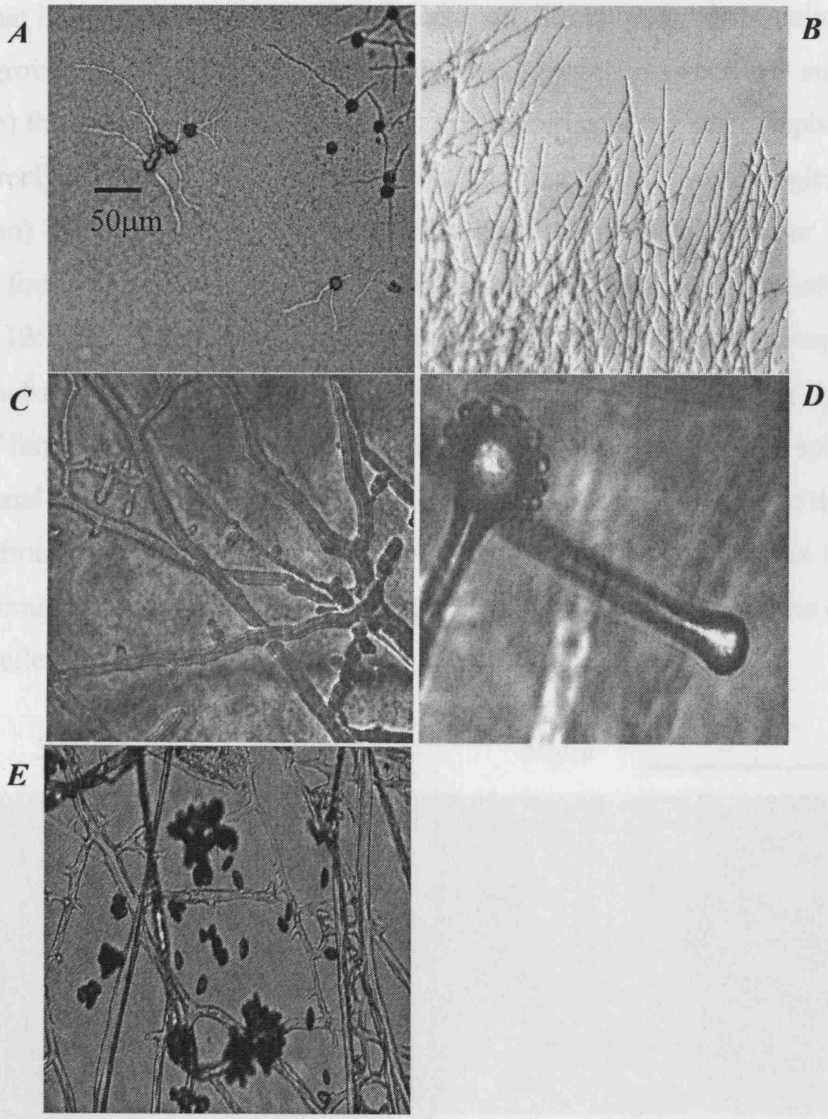


Figure 2.11: Light microscope images of *C. echinulata*. A: 4 hours growth, spores germinating B: 24 hours growth C: 48 hours growth D: 72 hours growth E: 96 hours growth-spores dispersed.

Growth of *C. echinulata* in submerged culture leads to a different growth characteristic from what has previously been observed. Growth during submerged culture gave rise to two growth forms, either pelleted or dispersed growth. At very low stirring speeds (<40rpm) the dispersed form is dominant. It is made up of branched hyphae which are either freely dispersed or form light 'fluffy' clumps. At higher agitation speeds (>100rpm) hyphae begin to clump and form pellets within a 12 hour period. This pelleted form consists of highly entangled hyphae which give rise to hollow spheres, figure 2.12: A&B. These spheres were viewed using an electron microscope and it was found that the outer layer of these spheres consists of radial growing hyphae which form a 'furry' coating. Under the naked eye the inner layers of the spheres appear smooth and hollow. However, electron microscope studies revealed that the pellets are formed from closely packed tangled hyphae, figure 2.12: C. Autolysis is thought to occur forming hollow spheres due to inefficient oxygen transfer into the centre of the fungal pellet.

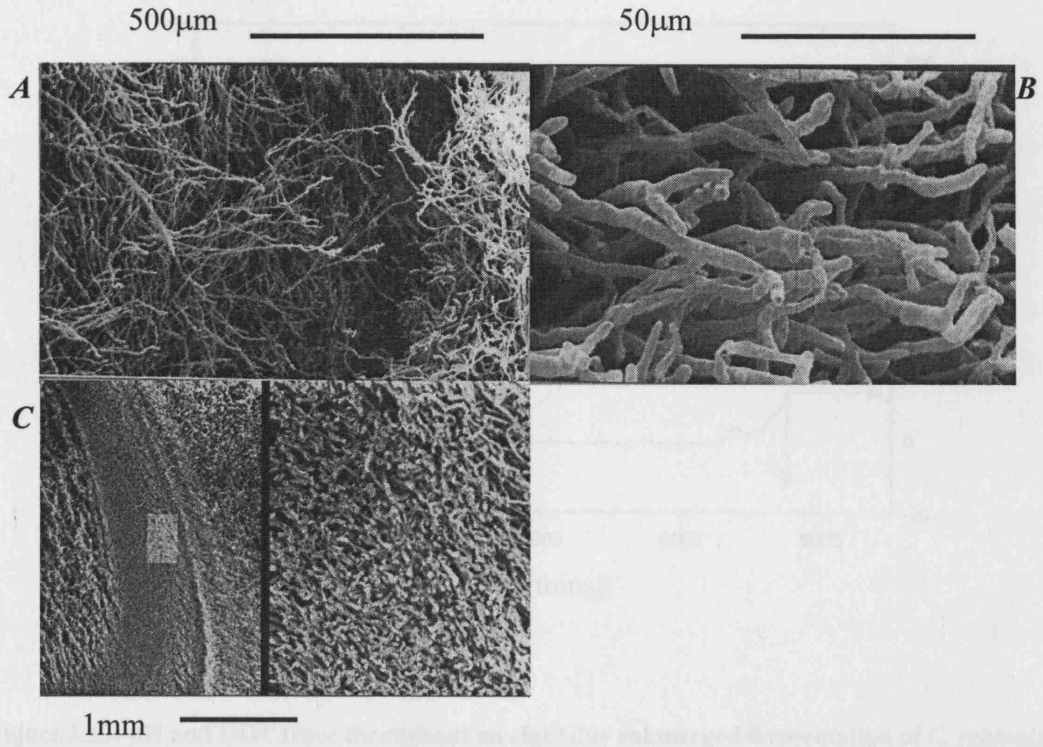


Figure 2.12: Electron microscope observations of *C. echinulata.* A: Outer layer of pellet (× 98.8) B: Outer layer of pellet (× 854) C: cut pellet, left hand image; inner pellet layer. Right hand image; pellet wall (× 26.8)

Throughout the growth of the fungus pH and dissolved oxygen tension (DOT) measurements were taken figure 2.13. During the course of the fermentation the DOT begins to decrease rapidly. This is followed by a sharp increase in pH which is maintained between pH 8-8.5 followed by a rapid increase in DOT. It is interesting to note that an increase in pH is only observed when DOT falls below 5%, usually occurring after three days growth.

This increase in pH signals the presence of BVMO within the fungus, only cells that have reached pH 8 -8.5 show BVMO activty. Harvesting cells before this pH increase has taken place yields cells with no BVMO activity, i.e. no bioconversion of (+/-) bicyclo[3.2.0]hept-2-en-6-one to lactone is observed.

BVMO activity within the fungal mass yielded 0.43 mg/ml 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone and showed very low amounts (~ 0.02 mg/ml) of corresponding 2-oxabicyclo[3.3.0]oct-6-en-3-one lactone (*cf* section 1.7, 20:1 ratio observed).

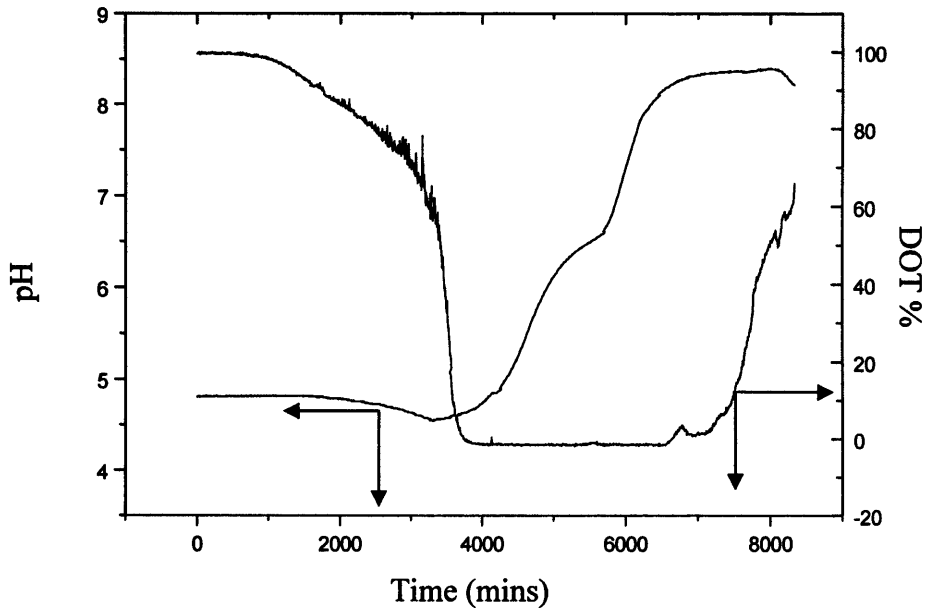


Figure 2.13: pH and DOT trace throughout an eight day submerged fermentation of *C. echinulata* DOT starting top left and pH starting bottom left.

Growth of the fungus on a large scale brought many problems and required several modifications to the fermenter. Baffles were removed as it was found that these caused pellet shearing along with 'clumping' of the fungal mass between the baffles and fermenter wall. Removing the baffles reduced the amount of clumping that occurred. However, stopping the fungal mass clumping around the sample tube and heating coil was not possible and therefore required disassembly of the fermenter after every run to allow cleaning by hand, increasing the turn around time between fermentations.

Due to the size of the pellets (*cf* 0.5 – 1.0 cm dia) harvesting via the small bore valve was impractical due to blockages. Therefore to overcome this problem a large bore needle (1.5cm dia) was used which was connected to a system of sterile water bottles to allow backwashing of the line if blockages occurred, therefore eliminating the risk of contamination.

To overcome the down time between each run, fermentations were often run in parallel thus allowing different conditions to be investigated.

The most obvious choices of conditions to alter were air rate, inoculum volume and stirring speed. pH was not altered in any of the experiments as this was the main indicator of BVMO.

It was found that high stirring speeds (450 rpm) were essential to maintain the small pelleted growth. At this speed, clumping and shearing was minimised. Lower speeds (200-300 rpm) resulted in large clumps of fungal mass.

Inoculum was taken from a static culture grown over 3 days. The concentration from each 500 ml culture was in the order of $1x10^8$ spores. Using two inoculum flasks gave the largest fungal mass at the end of the fermentation, approx 1-1.5Kg fungal pellets.

Air volume was also found to be a critical factor. At high air flow (10-15 L/min (0.66-1 vvm)) the DOT decreased to 50-60% followed by a sharp rise in pH, which maintained at values between 7-7.5 producing cells with no BVMO activity.

At air flows at 5 L/min (0.33 vvm) or less DOT rapidly decreased to 0% after 24 hours and slowly increased to values around 50%. pH slowly increased during the fermentation holding at values between 7 and 8 after eight to ten days. Cells grown at these conditions showed some BVMO activity and after 24 hours bioconversion yielded 0.2 g/L lactone.

At the end of the investigation it was concluded that the optimum air flow rate was at 7L/min (0.46 vvm). At this level DOT decreased after 2-4 days and initiated a rapid pH increase producing cells with BVMO activity.

It has been shown in this thesis that two distinct growth forms of *C. echinulata* exist, that of hyphal growth observed on plates or pelleted growth during submerged culture. Growth of *C. echinulata* on agar plates is well documented and this was the chosen method for maintaining stock cultures. However, little literature exists on the growth of this fungus during submerged culture. It was therefore essential to develop and understanding of the growth of *C. echinulata* during submerged culture if large quantities of fungal mass were to be obtained for later protein purification.

It was found that during submerged culture hollow pellets were formed consisting of highly branched entangled hyphae. However, no fruiting bodies were present in this growth form which was shown for the first time using electron microscopy.

The exact mechanism of pellet formation is not known and it is thought that the mechanism varies between species. However, two types of pellet forming organisms are recognised; coagulating and non-coagulating (Metz and Kossen, 1977). The coagulating type is where the spores coagulate in early stages of development and progress to form pellets. For the non-coagulating type a single spore has the ability to develop in to a pellet. The ability of the filamentous fungi to grow in its hyphal or pelleted form can be classified by: microscopic morphology, which determines the size and shape of hyphal elements and macroscopic morphology which determines the size and shape of the pellets (Nielsen and Krabben, 1995). Pelleted growth identical to *C. echinulata* has been studied in other fungi, particularly with *Aspergillus oryzae* and *Rhizopus nigricans* (Carlsen *et al.*, 1996, Agger *et al.*, 1998, Žnidaršič *et al.*, 1998).

Using these systems detailed growth patterns have been observed, such as hyphal growth rate, tip extension and coagulation of hyphae during culture. However, this has not been subject to study in this thesis as efforts were concentrated on obtaining sufficient quantities of fungal biomass for protein purification.

The cause of the pH increase is still largely unknown. However, in order to understand why a pH change may be occurring during the fermentation two basic chemicals, ammonium and nitrate were monitored. In figure 2.14, and it can be seen that ammonia levels peak when DOT reaches 0% and pH has risen to pH6. After 24 hours levels decrease abruptly and stabilise around 25mM until a final increase in DOT level where ammonium levels increase to 100 mM.

However, nitrate levels appear constant during low DOT with little increase towards the end of the fermentation.

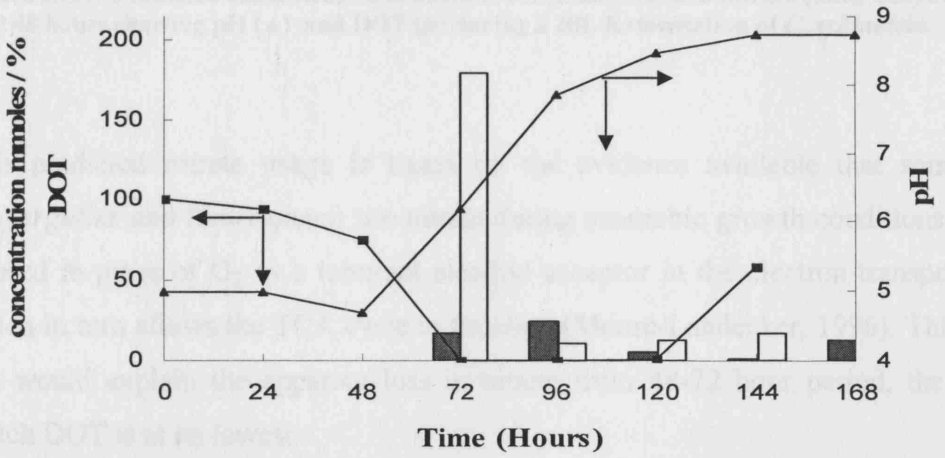


Figure 2.14: Concentration of ammonium (clear box) and nitrate (filled box) with pH (▲) and DOT (■) during a 20L fermentation of *C. echinulata*.

From figure 2.14 it can be seen that the levels of nitrate are relatively low at after 72 hours at 0% DOT and increasing pH conditions during the fermentation. Nitrate, as $NaNO_3$, is a component in the fermentation media and is added at a starting concentration of approx. 90 mM (NO_3). Using this value, it would be reasonable to plot a curve showing decreasing levels of nitrate over the first 48 hours figure 2.15.

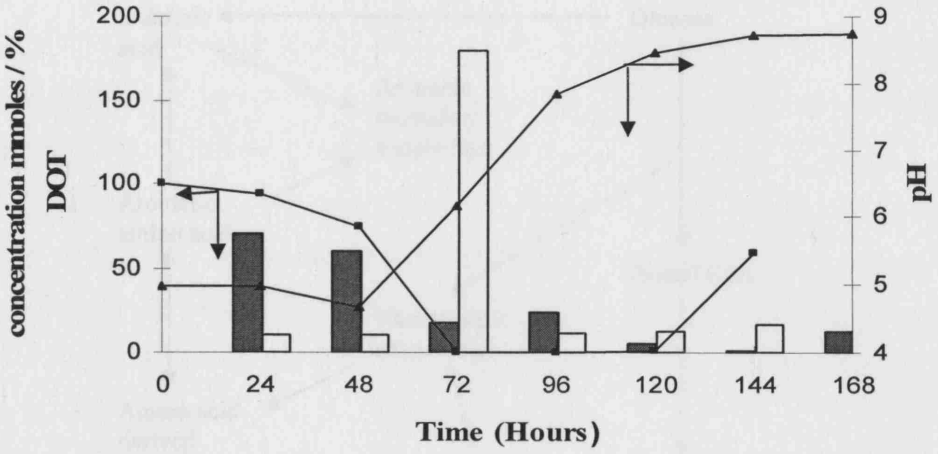


Figure 2.15: Predicted concentration of ammonium (clear box) and nitrate (filled box) over the first 48 hours showing pH (▲) and DOT (■) during a 20L fermentation of *C. echinulata*.

This predicted nitrate usage is based on the evidence available that some fungi (*Aspergullus* and *Neurospora*) use nitrate during anaerobic growth conditions. Nitrate is used in place of O_2 as a terminal electron acceptor in the electron transport chain which in turn allows the TCA cycle to function (Moore-Landecker, 1996). This nitrate use would explain the apparent loss in nitrate from 48-72 hour period, the time at which DOT is at its lowest.

During this time ammonium levels increase, derived from nitrogen catabolism, but decrease sharply after 96 hours. In fungi ammonium ions are used as a building block for further synthesis, for example amino acid synthesis where it is combined with α-ketoglutaric acid to form L-glutamic acid. These amino acid derived metabolites can be further converted to aromatic amino acids entering the Shikimic acid pathway resulting in the synthesis of aromatic secondary metabolites, figure 2.16.

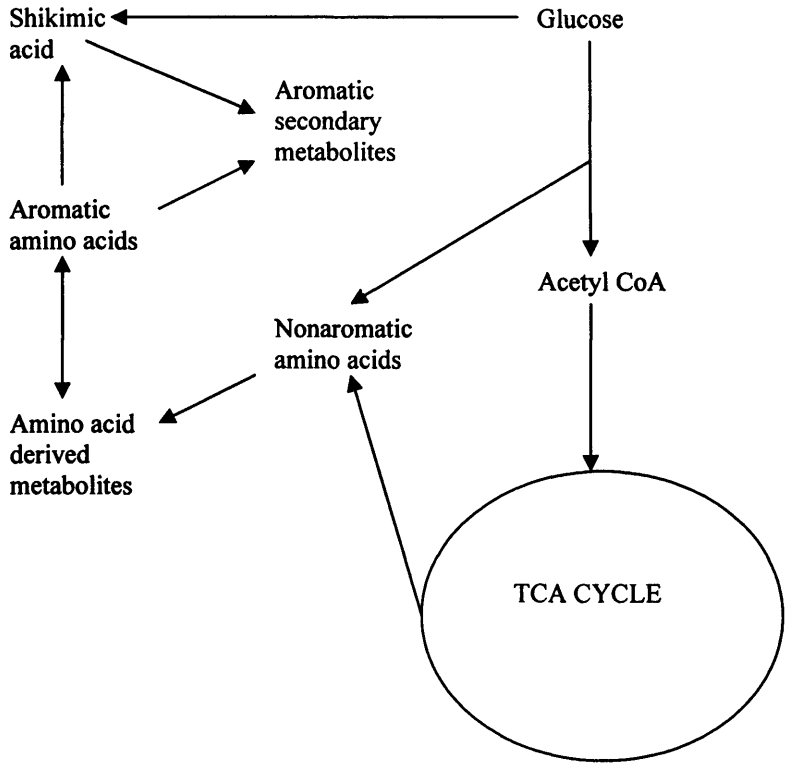


Figure 2.16: Schematic of TCA cycle linking in with other synthesis pathways

Fungi produce an extensive range of secondary metabolites for example, antibiotics and plant growth hormones, all of which are produced at the end of the growth phase under batch fermentation conditions and are not usually essential for growth. Most secondary metabolites are produced from metabolic intermediates. However, the enzymatic pathways used are encoded by specific genes which are usually species specific. The presence of the BVMO at the end of the culture fits within the current theory that secondary metabolism is essential in times of restricted growth conditions as a way of removing an excess of intermediates from the basic metabolic pathway.

Historically, growth of *A. calcoaceticus* on media containing cyclohexanol results in expression of the AcCHMO enzyme. This same methodology cannot be used in the case of *C. echinulata*. Unlike *A. calcoaceticus* which is a bacterium and therefore produces multiplying colonies displaying the expressed AcCHMO enzyme, *C. echinulata*, a fungus, produces fruiting bodies, as previously described. Experiments were run growing *C. echinulata* on media plates containing bicyclo[3.2.0]hept-2-en-6-ol and (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone, harvesting the fruiting bodies for the inoculum. No reduced expression time for the BVMO was observed in either sample.

As discussed later in section 5.3.3 thoughts surrounding the expression of the BVMO during submerged culture have been linked to a period of anaerobic growth during fermentation where it has been observed that the addition of alcohol to fungal cells grown for 24 hours induces the BVMO enzyme.

One possible theory on the inducement of the BVMO after this period of anaerobic fermentation is the increased levels of acetaldehyde present. This can be converted in to ethanol, enter the fatty acid cycle to produce methyl ketone or converted in to polyketides, complex secondary metabolites as outlined below in figure 2.17.

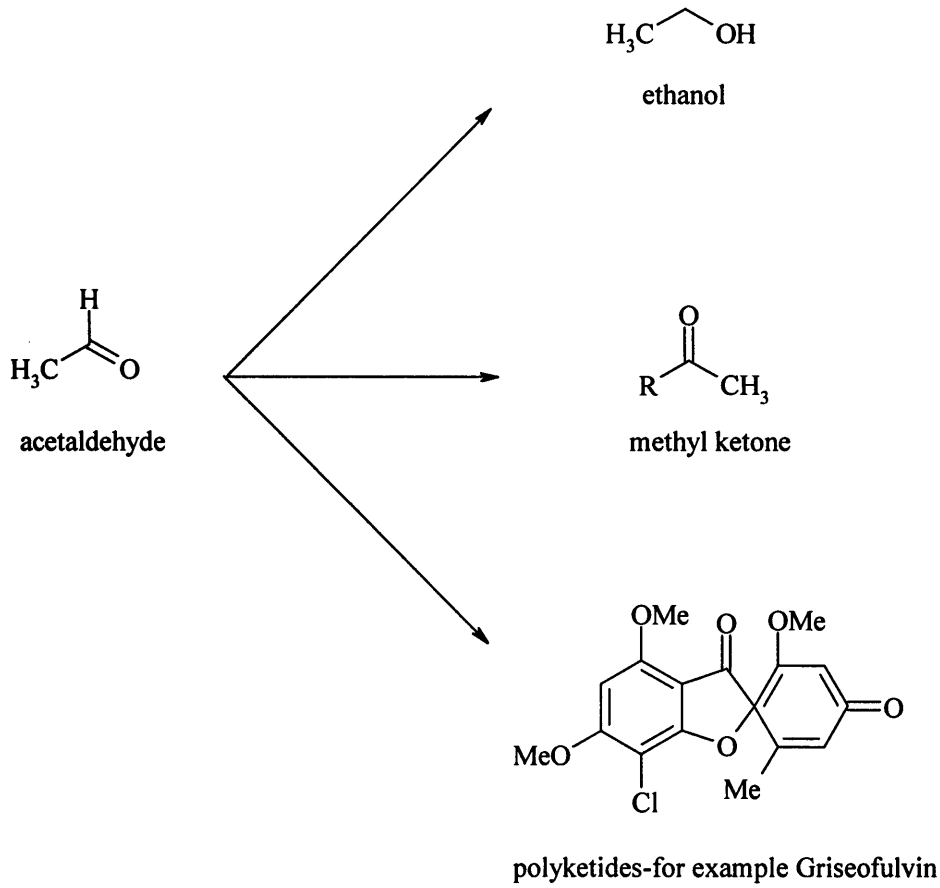


Figure 2.17: Possible products from the conversion of acetaldehyde

2.4.2 Substrate specificity

2.4.2.1 Activity and substrate inhibition studies using bicyclo[3.2.0]hept-2-en-6-one

A time course experiment on the transformation of (+/-) bicyclo[3.2.0]hept-2-en-6-one gave the following results (figure 2.18 and table 2.2). After the first 24 hour period a clear decrease in ketone concentration can be observed, with the biggest decrease seen at a concentration of 10 g/L, suggesting at this concentration the highest lactone production should be observed. However, lactone production was observed at low concentrations of ketone substrate, between 0.5 g/L and 4 g/L

At ketone concentrations above 4 g/L lactone is present in low yields. This would suggest that substrate inhibition occurs at concentrations above 4 g/L.

However, due to the volatility of the ketone the apparently large decrease seen at 10 g/L concentration is possibly due to losses from evaporation and not used for lactone production.

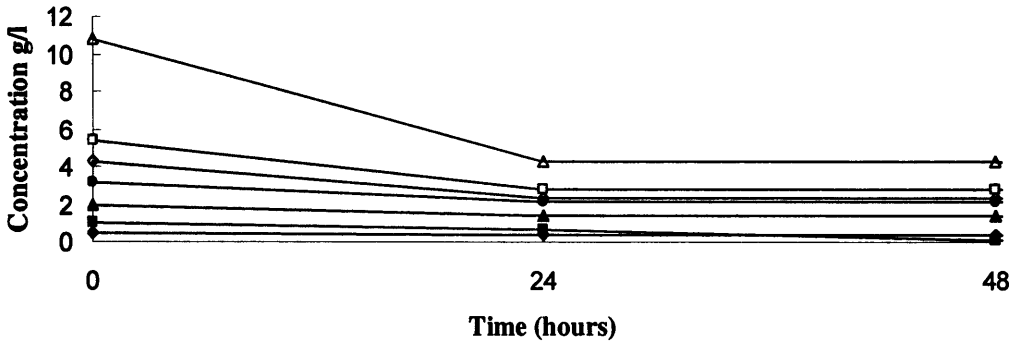


Figure 2.18: Consumption of bicyclo[3.2.0]hept-2-en-6-one ketone over 48 hours at the following concentrations: ♦ 0.5 g/L, ■ 1 g/L, ▲ 2 g/L, •3 g/L, ◊ 4 g/L, □ 5 g/L, △10 g/L.

Table 2.2: Lactone production over 48 hours at given bicyclo[3.2.0]hept-2-en-6-one ketone concentrations.

Ketone concentration g/L	Lactone concentration after 24 hours g/l	Lactone concentration after 48 hours g/l
0.5	0.20	0.20
1	0.09	0.09
2	0.14	0.14
3	0.03	0.03
4	0	0
5	0	0
10	0	0

Below in figure 2.19 the initial rate of reaction using *C. echinulata* has been plotted. From this graph it is clear that the highest rates can be achieved using a 0.5 g/L ketone concentration followed by a rapid decrease in initial activity at higher ketone concentrations resulting in inhibitory ketone values approaching 4 g/L when using the wild type BVMO in *C. echinulata.*

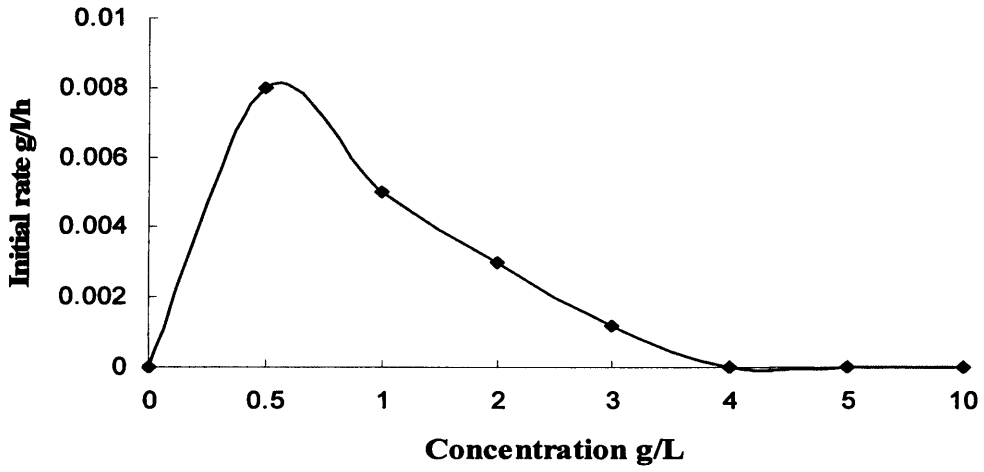


Figure 2.19: Initial rate of reaction as a function of ketone concentration using *C. echinulata*

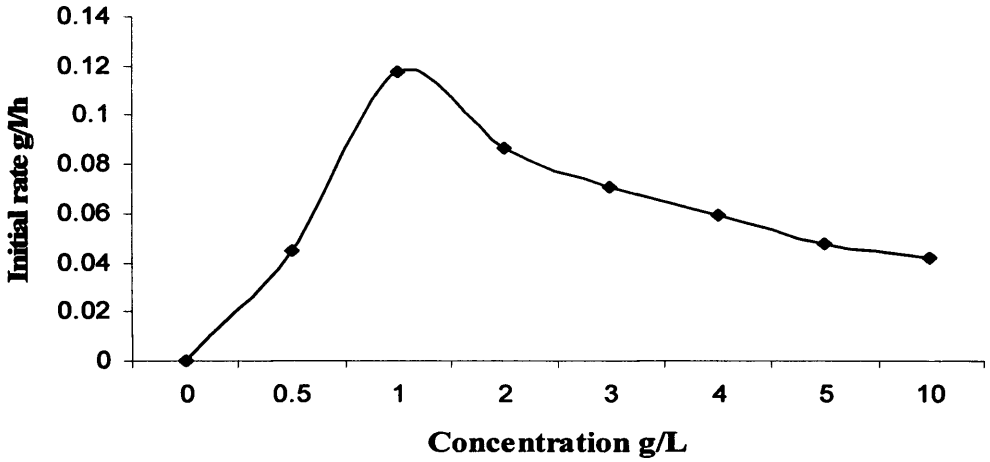


Figure 2.20: Initial rate of reaction as a function of ketone concentration using *E.coli* TOP10 pQR239

Figure 2.20 shows a plot of initial rate of reaction using *E. coli* TOP10 pQR329 where highest activity is observed at 1 g/L ketone concentration. However, it must be stressed that the initial rate of both the BVMO and CHMO was plotted using a 1 hour reaction run time.

Using these initial rate reaction plots has identified a similarity between the wild type BVMO and the over-expressed CHMO in terms of activity. It is clear that the over-expressed enzyme performs better than the wild type enzyme system, which of course is to be expected. However, the fact that the two enzyme systems have a similar reaction profile is interesting considering the enzyme source, fungal and bacterial.

2.4.2.2 Activity screening and inhibition studies using cyclic ketones

Organisms that contain a BVMO are known to perform reactions on a wide range of substrates. The substrates chosen were based firstly on ring size and followed with constant ring size with varying chain length.

This same set of substrates was also used with *E.coli* TOP10 pQR239 to provide a direct comparison with the behaviour of the BVMO.

Biotransformations were set up with *C. echinulata* using the selected ketones. However, the corresponding lactones were not observed, i.e. peaks not appearing where expected. Reactions were continued for a further five days after which decrease in ketone was observed in all samples, and unknown peaks were observed in the GC

traces suggesting that the ketones were undergoing breakdown in to products other than their expected lactones.

Figure 2.21 shows the results from substrate specificity using *E. coli* TOP10 pQR239 applying an identical range of ketones that were used with *C. echinulata* (Scarr *et al*, unpublished results).

It can be seen that the highest activity is observed using cycloheptanone and cyclohexanone (5.46 g/L/h and 5.36 g/L/h respectively) with the order of reaction as follows; cycloheptanone > cyclohexanone > 4-methyl-cyclohexanone > cyclobutanone > 4-propyl-cyclohexanone > cyclopentanone > 4-ethyl-cyclohexanone.

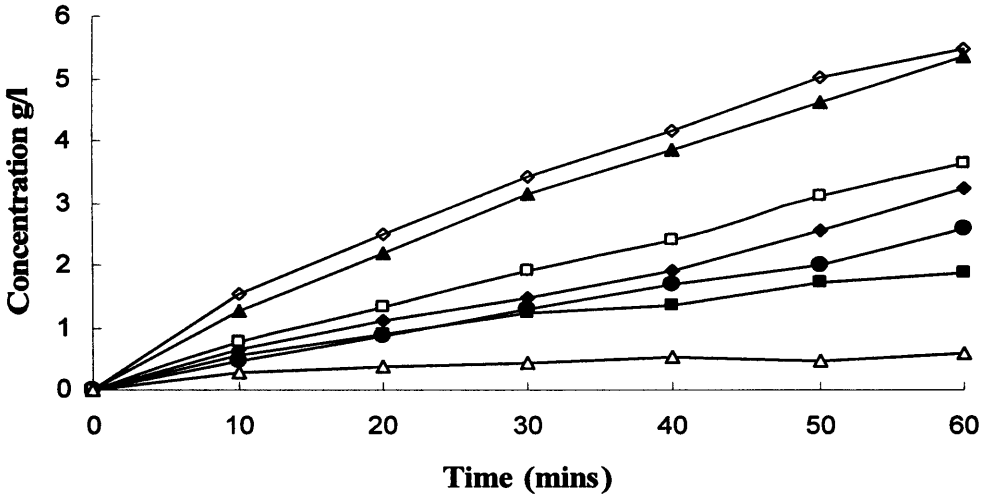


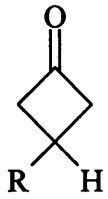
Figure 2.21: Lactone produced using *E. coli* TOP10 pQR239 CHMO with ketones at 1g/L ♦-cyclobutanone ■- cyclopentanone ▲-cyclohexanone ◊-cycloheptanone □-4-methy-lcyclohexanone △-4-ethyl-cyclohexanone ●- 4-propyl-cyclohexanone

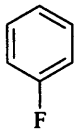
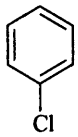
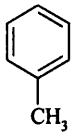
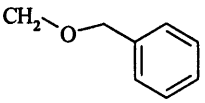
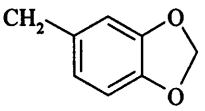
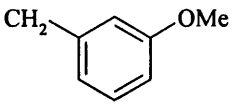
As shown *C. echinulata* does not show the ability to convert 4-substituted cyclohexanone substrates. However, studies carried out by Alphand and co-workers using 3-substituted cyclobutanones have shown that *C. echinulata* has the ability to convert some if not all that were studied (Alphand *et al.*, 1998), table 2.3.

It can be seen that the AcCHMO in *E. coli* has the ability to convert all of the ketones in to their corresponding lactones with most conversions > 80%. However, *C. echinulata* displays varying selectivity. It is interesting to note that the fungus appears to be able to accept large bulky R groups when combined with a small ketone ring group, in this case a four membered ring.

It is known that *A. calcoaceticus* has the ability to convert over 100 different substrates ranging in different ring sizes and groups (Stewart, 1998). It has become apparent that the wild type BVMO in *C. echinulata* does not display the same broad substrate specificity compared to *A. calcoaceticus* but appears specific for small membered rings displaying cyclobutanone structural motifs.

Table 2.3: Percentage yield of lactone from the bioconversions of 3-substituted cylobutanones using *C. echinulata* and *A. calcoaceticus* modified from Alphand *et al.*, 1998



R=	*C. echinulata* % yield	*A. calcoaceticus* % yield
	65	70
	80	89
	30	88
	4	73
	68	70
	68	83
	74	89
CH_2OtBu	25	43

2.5 Summary

The investigation of the fungus *C. echinulata* has revealed some fascinating insights in to the growth characteristics. It has been observed that growth during submerged culture results in pelleted growth and with the use of electron microscopy it was observed that these spheres consist of highly entangled hyphae with no fruiting bodies. Optimum growth conditions for a 15 L fermentation have successfully been developed providing kilogram quantities of fungal mass which will be used for protein purification.

Growth of *C. echinulata* on a large scale has exposed an interesting growth profile where both pH and DOT change rapidly throughout the fermentation process. This change in pH signals the presence of the BVMO and is thought to be brought about from the accumulation of secondary metabolites in the media resulting in an increased pH.

Inhibition studies using (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone have shown that optimum ketone concentration are between 0.5 and 1 g/L ketone with a rapid decrease in initial activity resulting in inhibitory ketone values approaching 4 g/L. However, the tolerance of the over expressed CHMO appears to be slightly higher with an optimum of 1g/L ketone concentration which decreases rapidly after this point.

Substrate specificity was carried out using a selection of cyclic ketones. However, *C. echinulata* failed to convert any into their corresponding lactones. The same set of cyclic ketones were applied to the recombinant CHMO and was shown to have an order of reaction as cycloheptanone > cyclohexanone > 4-methyl-cyclohexanone > cyclobutanone > 4-propyl-cyclohexanone > cyclopentanone > 4-ethyl-cyclohexanone. However, it has been shown that *C. echinulata* converts 3-substituted cyclobutanones.

CHAPTER 3

Protein purification of the Baeyer-Villiger monooxygenase

3.1 Introduction

To date the BVMO in *C. echinulata* has not been isolated. Ultimately, a cloned and over-expressed organism containing the CeBVMO is the final goal. However, low amounts of BVMO protein are expressed within the fungus. It is therefore necessary to grow on a large scale in order to obtain sufficient material for protein isolation. Growth studies, described previously in chapter two, have provided essential information on the growth characteristics that occur during the culture of this fungal organism, which has allowed successful growth on a 15L scale yielding approx 2Kg of material. In this chapter we focus on isolating the CeBVMO protein from the fungus with the intention of acquiring the N-terminus sequence which will allow degenerate primers to be designed. These primers will enable the BVMO DNA sequence to be captured and provide a platform for cloning in to a suitable alternate host organism.

Expressing the gene encoding this CeBVMO into an alternate host has always been a fundamental issue since the start of this project. The long growth time needed in order to generate viable fungal mass displaying CeBVMO activity makes the use of this wild type organism undesirable in commercial applications. It has been shown previously in chapter two that growth is not always successful and therefore cloning this enzyme in to an alternate host organism would be highly desirable allowing further exploration and understanding of this useful oxygenating enzyme system.

3.2 Materials and Methods

Unless stated otherwise all chemicals were of the highest purity available from Sigma-Aldrich Chemical Company (Poole, Dorset, U.K.). Growth media components were obtained from Oxoid Ltd (Basingstoke, Hants, U.K.). *Cunninghamella echinulata* NRRL 3655 was obtained from LGC (Teddington, Middlesex, U.K.).

3.2.1 Inoculum

As section 2.3.7.1

3.2.2 15L Fermentation

As section 2.3.7.2

3.2.3 BVMO activity assay

In a typical procedure 50 ml of fermentation media containing the fungal pellets was harvested directly from the fermenter aseptically. The fungal mass was harvested by filtration through a Miracloth membrane (20-22 μm pore size) (Calbiochem, Beeston, Nottingham, UK) and washed with 300 ml of 100 mM phosphate buffer (pH 8). 5 g of cells were re-suspended into 100 ml of 100 mM phosphate buffer (pH 8) and incubated with 100 μl (+/-) bicyclo[3.2.0]hept-2-en-6-one , in a reciprocating shaker at 170 rpm, 28°C for 24 hours.

3.2.4 Analytical Gas Chromatography (G.C.)

As section 2.3.4

3.2.5 Isolation of the BVMO enzyme from the fungal mass

Three different methods of protein extraction were used depending on the amount of fungal mass that was generated in order to attain the highest protein release. As a standard to all methods the fungal pellets from the 15 L fermentation were harvested by filtration through a Miracloth membrane and washed with 1.5 L of 100 mM sodium phosphate buffer (pH 8).

3.2.5.1 Extraction by sonication

5 g of fungal biomass was added to 10 ml 100 mM phosphate buffer (pH 8) and sonicated at 18 μ on ice, eight times for 30 sec periods with one minute rest between each sonication step. The suspension was then centrifuged (10,000g, 4°C, 30 min) and the supernatant decanted from the deposit. The supernatant was then concentrated using a centrifugal concentrator, 10KDa cut off (Vivascience Ltd, Epsom,U.K.)

3.2.5.2 Extraction by liquid nitrogen

5 g of fungal biomass was added to a cooled mortar containing liquid nitrogen and a sufficient amount of acid washed sand and glass beads (100 mesh BDH 15032) and ground for three minutes until a biscuit like texture was achieved. The ground fungal mass was then resuspended in 10 ml 100 mM phosphate buffer (pH 8). The suspension was then centrifuged (10,000g, 4°C, 30min) and the supernatant decanted from the deposit. The supernatant was then concentrated using a centrifugal concentrator (Vivascience Ltd, Epsom, U.K.)

3.2.5.3 Extraction by mechanical homogenisation

The fungal biomass (approx 1 Kg) was suspended into 3 L of 100 mM sodium phosphate buffer (pH 8). The fungal mass was then homogenised using a high pressure LAB 60 APV-Gaulin homogeniser (APV-Gaulin, Crawley, Sussex, UK) at one pass at 0 bar followed by two pass at 200 bar. The homogenised fungal mass was then centrifuged (5000g, 30 mins, 4°C) and the supernatant (approx 4 L) separated from the deposit and concentrated by tangential flow using 3 × 50 cm^2 10KDa cut off tangential concentrators (Millipore, Watford, UK). Ammonium sulfate was added slowly to the

concentrated supernatant with stirring at 4°C over 2 hours. The sample was centrifuged (10,000g, 60 mins, 4°C) and the deposit separated from the supernatant and made up in to 50 ml of 100mM sodium phosphate buffer (pH 8).

3.2.6 Bioconversion (+/-) bicyclo[3.2.0]hept-2-en-6-one using sonicated, homogenised and liquid nitrogen fungal biomass

A sample from each fungal biomass disruption was centrifuged at 10,000*g*, 4°C for 20 min and the supernatant decanted from the deposit.

10ml of supernatant obtained from centrifugation was added to 10 µl (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone and incubated in a reciprocating shaker at 170 rpm, 28°C for 24 hours.

5 g of each deposit obtained from centrifugation was added to 10 ml 100 mM phosphate buffer (pH 8) containing 10µl (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone and incubated in a reciprocating shaker at 170 rpm, 28°C for 24 hours.

3.2.7 Co-factor dependence

3.2.7.1 Preparation

Fungal biomass which had been disrupted by sonication was centrifuged (10,000g, 20 mins, 4°C) and the supernatant collected (approx. 15ml). This was further concentrated in an Amicon stirred cell concentrator (Millipore, Watford, U.K) using a 10 kDa membrane and further concentrated using a 10 kDa centrifugal concentrator yielding approx. 4ml concentrated supernatant.

3.2.7.2 Bioconversion setup

Three bioconversions were setup to test for co-factor dependence:-

Concentrated supernatant (0.5 ml) was added to 0.5 µl (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone with no co-factors present and incubated in a reciprocating shaker at 170 rpm, 28°C for 24 hours.

NADH (final concentration 100 mM) was added to concentrated supernatant (0.5ml) followed by the addition of 0.5 µl (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone and incubated in a reciprocating shaker at 170 rpm, 28°C for 24 hours.

NADPH (final concentration 100 mM) was added to concentrated supernatant (0.5ml) followed by the addition of 0.5 µl (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone and incubated in a reciprocating shaker at 170 rpm, 28°C for 24 hours.

3.2.7.3 Experimental controls

5 g of whole cell fungal biomass was added to 10 ml 100 mM phosphate buffer (pH 8) containing 10 µl (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone and incubated in a reciprocating shaker at 170 rpm, 28°C for 24 hours.

5 g of whole cell fungal biomass was added to 10 ml 100mM phosphate buffer (pH 8) and autoclaved at 121 °C. 10µl (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone was then added and the sample was incubated in a reciprocating shaker at 170 rpm, 28°C for 24 hours.

3.2.8 Ammonium sulphate precipitation

The supernatant and deposit obtained from centrifugation of fungal mass that had been disrupted by sonication (section 3.2.6.1) was subjected to a 80% ammonium sulfate precipitation.

5.61 g of enzyme grade ammonium sulfate was added slowly to 10 ml supernatant with constant stirring at 4°C over a period of four hours.

5.61 g of enzyme grade ammonium sulfate was added slowly to 10 ml 100 mM phosphate buffer (pH 8) and 5g deposit with stirring at 4°C over a period of four hours.

After four hours both samples were centrifuged at 12,000g, 4°C for 2 hours. The supernatant and deposit from the centrifugation were separated placed in to visking tubing (5KDa cut off) and dialysed for 24 hours in 100mM phosphate buffer (pH 8) (three changes). After 24 hours the samples were removed and bioconversions were performed as detailed below

1 µl (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone was added to dialysed supernatant (1 ml) containing 1 mg NADPH and incubated in a reciprocating shaker at 170 rpm, 28°C for 24 hours.
1 µl (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone was added to dialysed deposit (10 mg in 1 ml 100 mM phosphate buffer (pH 8)) containing 1mg NADPH and incubated in a reciprocating shaker at 170 rpm, 28°C for 24 hours.

3.2.9 Optimisation for bioconversions using sonicated biomass

Fungal biomass was disrupted by sonication as previously described (section 3.2.6.1) without centrifugation. 1 g of biomass was added to 10 ml of each different pH buffer (table 3.1) and 10 µl of (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone was added to each. The reactions were allowed to proceed for 24 hours in a reciprocating shaker at 28°C at 170rpm.

Table 3.1: Buffer compositions and concentrations used in pH optimisation experiments.

Buffer	Concentration mM	pH
Glycine + sodium hydroxide	50	9
Phosphate	50	8
Phosphate	50	7.2
Sodium succinate	50	5.5
Sodium acetate	50	5

3.2.10 The use of protease inhibitors and effects on BVMO activity

Fungal biomass was cultured as before (section 3.2.1). 1g of fungal pellets was disrupted by grinding in liquid nitrogen, sand and glass beads (section3.2.6.2) with 5ml of each different protease inhibitor until a biscuit like texture was achieved. The resulting solution was centrifuged (2,000g, 4°C, 10mins) and the supernatant and deposit separated.

The protease inhibitors used are listed below in table 3.2. and used following recommendations from Sigma-Aldrich Co Ltd.

Table 3.2: Protease inhibitors used during protein extraction.

Inhibitor	Concentration mM
AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride)	1
E-64 (trans-epoxysuccinyl-L-leucyamido(4-guanidino)butane)	0.14
Pepstatin A	0.05
1,10-phenathroline monohydrate	5
2,2-dithodipyridine	10
Alloxan	10

3.2.10.1 Bioconversion reactions

The supernatant and deposit were made up in 1 ml 100 mM phosphate buffer (pH 8) containing 1 mg NADPH. 1 µl (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone was added and incubated in a reciprocating shaker at 170 rpm, 28°C for 24 hours after which they were assayed by GC.

3.2.11 Inhibiting esterase activity using protease inhibitors

Fungal biomass which had been disrupted by sonication (section 3.2.6.1) was centrifuged (10,000g, 20 mins, 4°C) and the supernatant collected. This was further concentrated in an Amicon stirred cell concentrator using a 10 KDa membrane (Millipore, Watford, U.K.)

Alloxan was prepared at 10 mM, E64 was prepared at 14 μM/ml and AEBSF at 1 mM. The general esterase substrate *para*-nitrophenyl acetate (pNPHOAc) was used to monitor the lactone hydrolase activity. pNPHOAc was prepared as a 10 mM stock in ethanol. All readings were taken at 348 nm (UVicam AT-UV2, Univacm Ltd, Cambs, UK) as this represents the isobestic point, the wavelength at which the absorbance is pH independent. Reactions were set up as detailed below:

Substrate blank

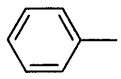
100 μl +10KDa cell extract was added to 800 μl 100 mM sodium acetate buffer (pH 6) followed by the addition of 100 μl ethanol (in place of the pNPHOAc)

Inhibitor assay

100 μl +10KDa cell extract was added to 800 μl 100mM sodium acetate buffer (pH 6) followed by the addition of 100 μl inhibitor and left at room temperature for 30mins after which 100 μl pNPHOAc was added and absorbance readings taken.

3.2.12 HiTrap Hydrophobic Interaction Chromatography Columns (HIC)

HiTrap 1ml column selection kit was obtained from Amersham Pharmacia Biotech.

Phenyl Speharose high performance	Highest ionic strength	
Octyl Sepharose		CH_3-$(CH_2)_6$-CH_2-
Butyl Sepharose	Lowest ionic strength	CH_3- CH_2- CH_2- CH_2- CH_2-

Buffers used:-	High Salt	100 mM Tris HCl (pH 8), 1M Ammonium Sulfate
	Low Salt	100 mM Tris HCl (pH 8)

The column was equilibrated with high salt buffer (10 ml). A 0.5ml protein sample (from 80% ammonium sulphate precipitation) was then loaded on to the column followed by a high salt wash (10 ml) and low salt wash (10 ml). Fractions were then collected from each wash and run on a 12.5% SDS-PAGE gel and analysed by G.C.

3.2.13 HiTrap Ion Exchange Chromatography Columns (IEX)

HiTrap 1 ml column selection kit was obtained from Amersham Pharmacia Biotech.

DEAE Sepharose Fast Flow
Q Sepharose

Buffers used:-	Low Salt	100 mM Tris HCl pH 8
	High Salt	100 mM Tris HCl pH 8 , 1.0M NaCl

5 ml ammonium sulfate suspension (from 80% precipitation) was dialysed (MWCO 8KDa, Medicell International Ltd, London, UK) for 24 hours with 3 changes of 100 mM Tris HCl buffer (pH 8).
The column was equilibrated with low salt buffer (10 ml). A 0.5 ml protein sample was loaded on to the column followed by a low salt was (10 ml) and finally a high salt wash

(10 ml). Fractions were then collected and run on a 12.5% SDS-PAGE gel and analysed by G.C.

3.2.14 Dye Resin Affinity Chromatography Columns

5ml columns containing Reactive Red 120 Agarose Type 3000-CL and Cibacron Blue 3GA Agarose Type 3000-CL were used.

Buffers used:-	High Salt	25 mM sodium phosphate buffer (pH 8) 1.0 M NaCl
	Low Salt	25 mM sodium phosphate buffer (pH 8)

5 ml ammonium sulfate suspension (from 80% ammonium sulphate precipitation) was dialysed (MWCO 8KDa, Medicell International Ltd, London, UK) for 24 hours with 3 changes of 100 mM sodium phosphate buffer (pH 8). The column was equilibrated with low salt buffer (10 ml) for 24 hours at 4°C. A 5 ml protein sample was loaded on to the column and left to equilibrate for 24 hours at 4°C followed by a high salt wash (10 ml). Fractions were collected and run on a 12.5% SDS-PAGE gel and analysed for BVMO activity by G.C.

3.2.15 Polyacrylamide Gel Electrophoresis PAGE

Routine protein identification was carried out using denaturing 12.5% and 19.5% SDS-PAGE acrylamide gels (Bio-Rad Laboratories Ltd, Hertfordshire, U.K.)

3.2.15.1 Stock solutions for SDS-PAGE gel

Acrylamide solution: 29.2% [*w/v*] acrylamide, 0.8% [*w/v*] N,N'-methylene bisacrylamide in water (Bio-Rad Laboratories, Ltd).

Separating gel buffer: 1.5 M Tris HCL, (pH 8.8)

Stacking gel buffer: 0.5 M Tris HCL, (pH 6.8)

Electrode solution: 0.05 M Tris HCL, 0.38M Glycine, 0.1% [*w/v*] SDS; adjusted to pH 8.8

SDS solution: 10% [*w/v*] SDS in double distilled Purite® water

Ammonium persulfate solution: 10% [*w/v*] ammonium persulfate in double distilled Purite® water

Sample buffer: 125 mM Tris HCL, (pH 6.8), 2% [*w/v*] SDS, 20% [*v/v*] glycerol, 0.001% [*w/v*] Bromophenol Blue tracking dye, 0.005% [*v/v*] β-mercaptoethanol.

Staining solution: 0.05% [*w/v*] Coomassie Brilliant Blue, 50% [*v/v*] methanol, 10% [*v/v*] acetic acid in double distilled Purite® water.

3.2.15.2 Preparation of SDS-PAGE gels

The gels were assembled according to the manufacturers instructions.

The separating and stacking gel were prepared as detailed below in table 3.3.

Table 3.3: Preparation of an SDS PAGE gel.

Component	12.5% Gel		19.5% Gel	
	Separating gel (ml)	Stacking gel (ml)	Separating gel (ml)	Stacking gel (ml)
Acrylamide Solution	4.2	2	6.5	3.0
Separating gel buffer	2.5	0	2.5	0
Stacking gel buffer	0	2.5	0	2.5
SDS solution	1	1	1	1
Distilled water	2.3	4.5	0	3.5
Ammonium persulfate	100µl	100µl	100µl	100µl
TEMED (N,N,N',N'-tetramethy-lethylendiamine)	10µl	10µl	10µl	10µl

The separating gel was poured first and allowed to set followed by the stacking layer gel along with a well forming comb inserted in to the top of the gel. After polymerization the comb was removed and the gel was inserted in to the electrophoresis apparatus following the manufactures instructions.

3.2.15.3 Preparation of samples

Samples were mixed with sample buffer (20µl equivolume) and heated to 100° C to denature the protein. A 20 µl sample was loaded on to the gel along with 5µl broad range markers for sample weight comparison (Bio-Rad Laboratories Ltd). The markers represent 15, 25, 37, 50, 75, 100, 150 and 250 kDa with the 50 kDa band being a heavier reference band. The samples were run at 200V (constant) until the dye front reached the bottom of the gel, approximately one hour.

3.2.15.4 Staining procedure

Gels were visualised by staining with Coomassie Brilliant Blue stain. The gel was placed in to a 1L glass beaker and covered with 50ml staining solution and heated in a microwave on full power for 3 mins. The stain was removed and the gel was heated for a further 10 mins in 500ml distilled water.

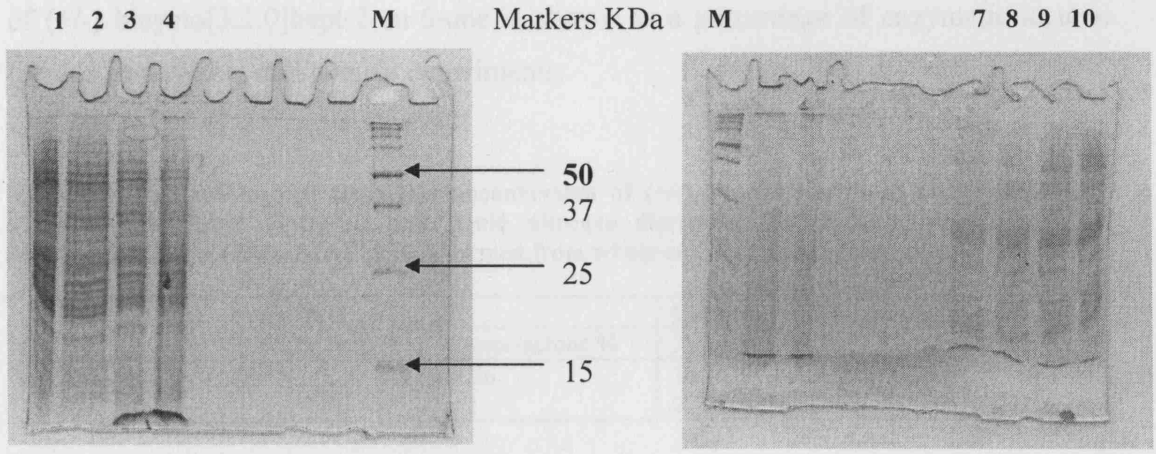
3.3 Results

3.3.1 Isolation of the BVMO enzyme from the fungal mass

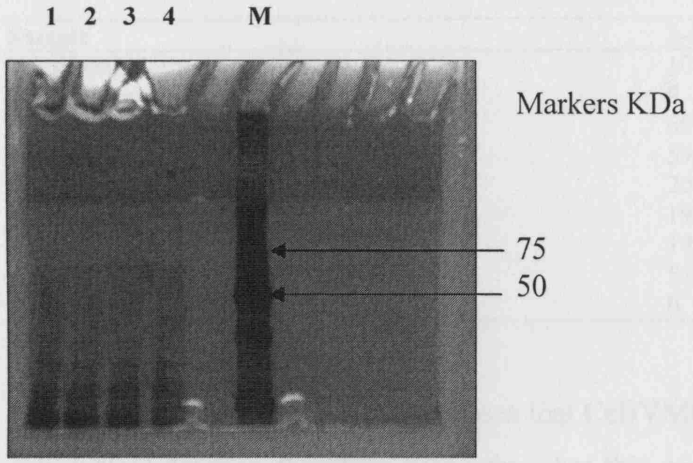
In order for the CeBVMO to be isolated an efficient method of cell disruption must be determined. Three different methods of protein disruption were used, disruption by sonication, disruption by liquid nitrogen and finally using mechanical homogenisation. From the SDS-PAGE gels (figure 3.1) it can be seen that the fungal mass is sufficiently disrupted in all three methods employed releasing proteins over a broad molecular range.

Protein bands were observed in the deposit after sonication but this was not observed in the deposit from the liquid nitrogen extraction suggesting that extraction by liquid nitrogen is a more efficient method. This method of protein extraction could further be improved by adding a sufficient quantity of acid washed sand and glass beads to the liquid nitrogen. This improved method ensures that all of the fungal mass is fully disrupted providing maximum protein release. However, this extraction method is only useful when processing small quantities of (approx 500g) fungal mass, using bigger quantities reduces the efficiency and this method was not always consistent and reproducible.

When dealing with large quantities of fungal mass produced from a 15L fermentation (approx 2Kg), sonication and liquid nitrogen extraction are not suitable methods. In this instance material must be processed by mechanical homogenisation. As described earlier in section 3.2.5.3 the disruption process involved passing the fungal biomass through the homogeniser first at 0 bar pressure followed by two passes at 200 bar pressure. The disruption process passes material though a small bore aperture and the force at which material flows though can be controlled up to a pressure of 500 bar. Due to the size of the fungal pellets (approx 0.5-1cm diameter), on the initial pass through the small bore aperture the pellets were broken open. Two further passes at a higher pressure ensured that the pellets were fully disrupted. Samples taken from the homogeniser after each pass were run on a gel. The concentration of protein on the gels is relatively low as the homogenate was in a total volume of 3 L buffer therefore gel silver staining was used to visualise proteins.



Gel 1 **Gel 2**



Gel 3

Figure 3.1: SDS-PAGE gels showing protein release from three different extraction methods. Gel 1- liquid nitrogen extraction: M= markers, 1-4= concentrated protein with increasing quantities of loading buffer. Gel 2- sonication: M= markers, 7-10= concentrated protein with increasing quantities of loading buffer. Gel 3- extraction by mechanical homogenisation

Bioconversions were set up using protein from all three extraction methods and BVMO activity was recorded (table 3.4 and table 3.5). Lactone formed from the bioconversion of (+/-) bicyclo[3.2.0]hept-2-en-6-one is shown as a percentage of enzymatic lactone formed from whole cell control experiments.

Table 3.4: Lactone formed from the bioconversion of (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone using material from sonication and liquid nitrogen disruption shown as a percentage of 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone formed from whole cell control experiment.

	Sonication		Liquid nitrogen extraction	
	3-oxa lactone %	2-oxa lactone %	3-oxa lactone %	2-oxa lactone %
Supernatant	26	0.26	18	0.3
Deposit	0	12	0	8

Table 3.5: Lactone formed from the bioconversion of (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone using material from mechanical homogenisation disruption shown as a percentage of enzymatic lactone formed.

Sample	3-oxa lactone %
Whole cell biotrans	100
Pass 1 supernatant (0 bar)	0
Pass 2 supernatant (200 bar)	60
Pass 3 supernatant (200 bar)	59
Pass 1 deposit	20
Pass 2 deposit	19
Pass 3 deposit	17
Concentrated pass 3 (conc 15X)	51
Waste from conc pass 3 sample	0

From the data in table 3.5 it can be seen that CeBVMO activity is predominantly in the supernatant fraction therefore suggesting that this protein is not membrane bound. A higher percentage of enzymatic lactone is observed in the sonicated fraction compared to that of disruption by liquid nitrogen. Activity is seen in the deposit from both disruption methods which is likely to arise from CeBVMO protein that has not been fully disrupted.

Low yields of enzymatic lactone are apparent in both samples. This low activity may be due to protein being lost or denatured during the disruption process for example, during sonication heat is produced and it is therefore important to keep the sample as chilled as possible to prevent total denaturation. When performing disruption by liquid nitrogen the initial stages of disruption are performed at -100°C. However, the sample warms up to room temperature quickly and goes though several freeze thaw steps which inevitably denatures a significant fraction of proteins.

The activity after each pass of mechanical homogenisation can be clearly seen with most CeBVMO active protein being disrupted after the second pass (table 3.5). Around 20% activity is observed in the deposit which decreases with each pass which would suggest that some CeBVMO active protein is not fully disrupted from the cells. However, in one experiment further passes were used and it was found that after 3 passes no significant increase in CeBVMO active protein was observed in fractions which had undergone 3 passes or more. It must also be noted that the above bioconversions were run with the addition of NADPH cofactor. At the time of the large scale experiments it was already found that performing bioconversion using cell free extract required the addition of NADPH.

3.3.2 Co-factor dependence

Table 3.6 shows the results from the cell free bioconversion of (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone in the presence of the cofactors NADH and NADPH. The results suggest that the CeBVMO enzyme is NADPH dependent, as enzyme induced 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone was produced in a higher yield (61%) compared to that of the bioconversion where NADH was used giving a 28 % conversion. However, it must be noted that this experiment has limitations with regards to removing all non bound co-factors, as observed in the 20% conversion seen when using supernatant and no co-factors. Some activity is observed using NADH. However, this may be due to a different enzyme which has the ability to convert (+/-) bicyclo[3.2.0]hept-2-en-6-one or the action of a nicotinamide nucleotide transhydrogenase enzyme conversion of NADH into NADPH. Overall, it would appear that NADPH is the preferred co-factor.

Table 3.6: Lactone formed from the bioconversion of (+/-) bicyclo[3.2.0]hept-2-en-6-one using material from sonication disruption and the addition of NADH and NADPH cofactors.

	3-oxa lactone %	2-oxa lactone %
Control whole cell bioconversion	100	0
Supernatant from sonication no co-factors	20	0
Supernatant from sonication + NADH	28	0
Supernatant from sonication + NADPH	61	0
Autoclaved whole cell	0	0

3.3.3 Ammonium sulphate precipitation

An ammonium sulfate cut was performed to 80% saturation using fungal biomass that had been disrupted by sonication. Bioconversions were run and the data is displayed below in table 3.7

Table 3.7: Lactone formed from the bioconversion of (+/-) bicyclo[3.2.0]hept-2-en-6-one using sonicated material which has been subjected to an 80% ammonium sulfate precipitation.

Ammonium sulfate fraction	3-oxa lactone %
Ammonium sulfate pellet	94
Supernatant	2.96

From the data it is clear that most of the active CeBVMO protein can be precipitated out using 80% ammonium sulfate. However, this method of crashing out protein is not selective at this concentration and will inevitable crash out most, if not all proteins in solution. The methodology behind this experiment was to preserve the CeBVMO activity which in turn would allow purification of the CeBVMO enzyme which is discussed later in section 3.3.6.

3.3.4 pH Optimisation for biotransformations using sonicated biomass

From the data (table 3.8) it can be seen that CeBVMO activity in cell free has an optimum pH between 7 and 8 yielding > 90% enzyme induced lactone (3-oxabicyclo[3.3.0]oct-6-en-2-one) at this range whereas in the case of the whole cell system there was a broad pH optimum (6-8), as reported previously (Alphand *et al.*, 2000).

Table 3.8: Lactone formed from the bioconversion of (+/-) bicyclo[3.2.0]hept-2-en-6-one at different pH conditions using sonicated material.

Sample	3-oxa lactone %	2-oxa lactone %
Whole cell biotransformation	100	1.8
pH 9 50mM	68	0
pH 8 50mM	96	8
pH 7.2 50mM	92	10
pH 5.5 50mM	88	0
pH 5 50mM	70	6

3.3.5 The use of protease inhibitors and effects on BVMO activity in cell free extract

From the previous experiments it has become clear that the CeBVMO activity converting (+/-) bicyclo[3.2.0]hept-2-en-6-one to lactone is severely reduced when the fungal cells are broken open. Enzymes which were once compartmentalised in the whole cell are now free during the bioconversion reactions. It is known that esterase enzymes have the ability to cleave lactone ring products and therefore it is highly likely that these enzymes are ring opening the lactones formed from this bioconversion giving rise to low yields compared to that in the whole cell reaction. It is well documented that *A. calcoaceticus* contains a lactone hydrolase enzyme which was part of the AcCHMO pathway, therefore the presence of a similar enzyme in *C. echinulata* comes as no surprise. This experiment was used to investigate the effects of protease inhibitors on the BVMO enzyme and to observe any changes in the amounts of lactone formed during cell free bioconversion.

Table 3.9: Lactone formed from the bioconversion of (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone with the addition of inhibitors and NADPH using sonicated material.

Inhibitor	Enzymatic lactone %
Whole cell biotransformation	100
Alloxan	56
Dithiodipyridine	15
AEBSF	25
E64	36
Pepstantin A	53
No inhibitor	10

From the data shown in table 3.9 it can be seen that the amount of lactone produced is significantly higher when inhibitors are used compared to that when no inhibitor is present. This suggests that the inhibitors have no direct affect on the CeBVMO enzyme but directly affect a lactone hydrolase type enzyme present in the cell free extract yielding greater amounts of lactone. It can be seen that the addition of Alloxan, Pepstantin and E64 to the cell free extract yield the highest amounts of lactone compared to using cell free extract with no inhibitor.

To confirm that the presence of a lactone hydrolase and the effect it has on the lactones produced a sample of crude cell free extract obtained from sonication was applied to the media from a whole cell bioconversion that was known to contain 0.5 mg/ml enzyme induced lactone (3-oxabicyclo[3.3.0]oct-6-en-2-one). After 24 hours the enzyme

induced lactone had been reduced to 0.4410 mg/ml clearly indicating the presence of this lactone hydrolase.

From these initial results, further investigation in to the use of these inhibitors to inhibit the lactone hydrolase have reviled that E64 and Alloxan were the best candidates. Table 3.10 shows the effects these inhibitors have on the lactone hydrolase.

Table 3.10: Lactone hydrolase activity monitored by the conversion of *para* nitrophenyl acetate to *para* nitrophenyl.

Inhibitor	Enzyme conversion µmol/min/ml
No inhibitor	7.24
Alloxan 10mM	1.4
E64 14µM	0.8
AEBSF 1mM	3.9

The data shown in table 3.10 measures the activity of the lactone hydrolase by monitoring the conversion of para-nitrophenyl acetate (colourless) to para-nitophenyl (yellow). It can be seen that E64 has the highest enzyme inhibition followed by Alloxan. If subsequent purification of the CeBVMO is to be successful, monitoring the CeBVMO activity must be reliable. It was therefore decided that Alloxan would be added to all further free cell bioconversions. However, E64 was not chosen despite giving the best enzyme inhibition, due to cost implications and availability.

3.3.6 Development of protein purification protocol

In order to asses the best and most efficient method of purifying the CeBVMO protein a range of test columns were used. These were either hydrophobic interaction, ion exchange or dye ligand exchange based. Out of the five different hydrophobic interaction columns that were tested butyl sepharose resulted in the protein binding to the column. However, using this column gave rise to many proteins in the active fraction which would increase the number of purification steps that would be necessary to isolate the CeBVMO. In this case using phenyl sepharose high substitution where the protein was eluted in the high salt wash, i.e. no binding to the column occurred, yielded fewer proteins in the active fraction therefore, reducing the number of purification steps required.

Table 3.11: Separate column activity tests using crude isolated protein.

Column	Fraction	3-oxa lactone %
Phenyl Sepharose high sub	High salt	93
Phenyl Sepharose high sub	Low salt	8
Q Anion exchange	Low salt	83
Q Anion exchange	High salt	17
Reactive Red	High salt	64
Reactive Red	Low salt	29
Reactive Blue	High salt	28
Reactive Blue	Low salt	63

It can be seen above in table 3.11 that the CeBVMO protein binds to the reactive red dye ligand column but not the Cibacron blue column. These dye columns work on the principle that the dye structures are similar to those of nicotinamide cofactors. In the case of the Cibacron blue column the aromatic triazine dye structure is similar to that of nicotinamide adenine dinucleotide (NADH) whereas the Reactive Red dye structure is similar to that of nicotinamide adenine dinucleotide phosphate (NADPH).

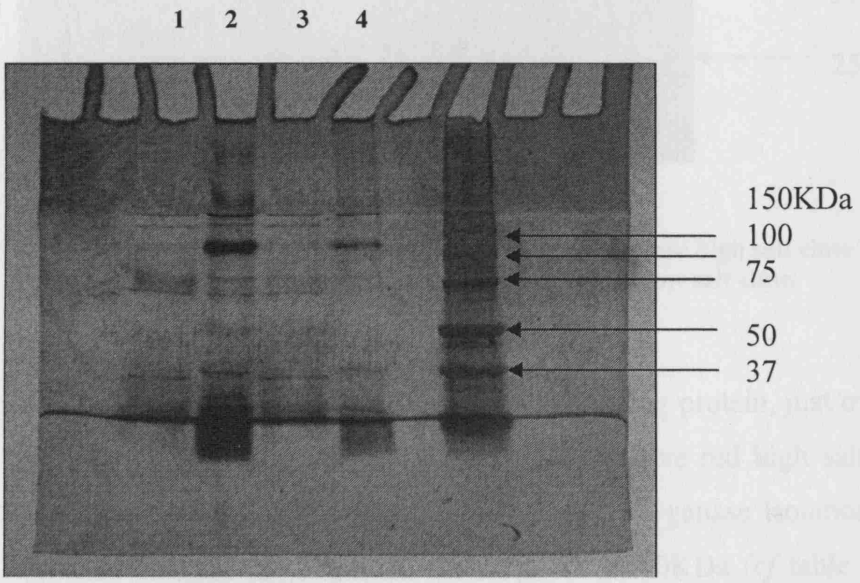


Figure 3.2: SDS PAGE gel silver stained. 1- Reactive Red high salt elute 2- Reactive Red low salt elute. 3- Reactive Blue high salt elute 4- Reactive Blue low salt elute.

Table 3.12: Activity assay using active fraction from phenyl sepharose purification followed by separation using Reactive Red 120 column.

Column	Fraction	3-oxa lactone %
Phenyl Sepharose high sub	High salt	93
Reactive Red 120	High salt	105
Reactive Red 120	Low salt	0

Table 3.12 shows the initial purification steps using phenyl sepharose followed by Reactive Red 120. All fractions were run on a SDS PAGE gel and the results are shown below in figure 3.3.

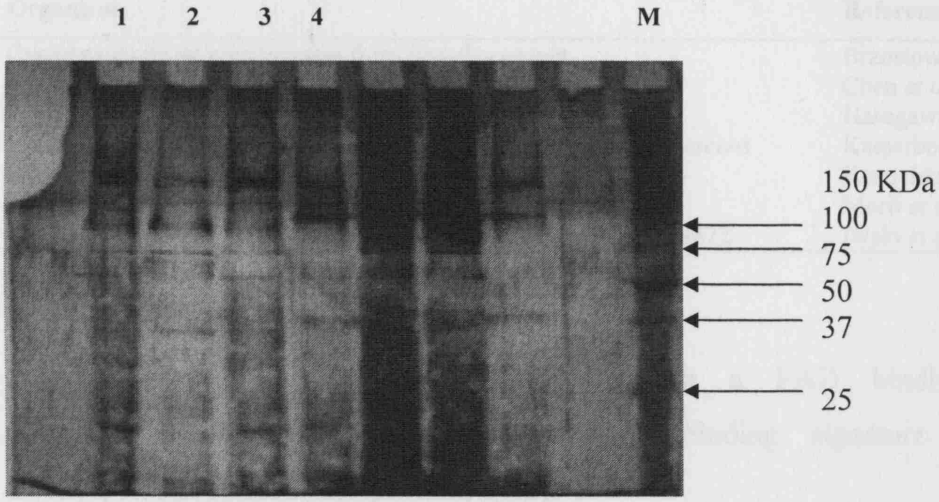


Figure 3.3: SDS PAGE gel silver stained. 1-phenyl sepharose high salt elute 2- phenyl sepharose low salt elute 3- Reactive Red high salt elute 4- Reactive Red low salt elute.

From figure 3.3 it can be seen that a corresponding protein, just over 50KDa is present in both the phenyl sepharose high salt and reactive red high salt fractions where the activity was highest. Based on previous monooxygenase isolations where monomeric proteins isolated have been in the range of 50-70KDa (*cf* table 1.3, section 1.3.3) it would appear that this protein at 50KDa is a promising target for the fungal CeBVMO.

Ultimately, the aim was to isolate the CeBVMO protein and obtain sequence data so that cloning and over expression could be carried out.

In order to reach the goal of obtaining the CeBVMO sequence data this project has taken several routes. The first route, and most discussed in this thesis was to gain an understanding of the growth of the fungus allowing subsequent protein purification via interaction chromatography. However, a second route focusing on the DNA sequence data was also briefly touched on towards the end of the project.

Current monooxygenases that have been isolated and cloned providing valuable sequence data are detailed in table 3.13.

Table 3.13: BVMO that have been cloned and sequenced

Organism	Reference
Cyclohexanone monooxygenase from *Brevibacterium*	Brzostowicz *et al.*, 2000
Cyclohexanone monooxygenase from *A. calcoaceticus*	Chen *et al.*, 1988
Cycohexanone-1,2-monooxygenase from *Exophiala jeanselmei*	Hasegawa *et al.*, 2000
Hydroxyacetophenone monooxygenase from *Pseudomonas fluorescens*	Kamerbeek *et al.*, 2001
Cyclodecane monooxygenase from *Rhodococcus ruber*	Kostichka *et al.*, 2001
Steroid monooxygenase from *Rhodococcus rhodochrous*	Morii *et al.*, 1999
Cyclopentanone monooxygenase from *Comamonas* sp. NCIMB 9872	Iwaki *et al.*, 2002

It has been found that all BVMOs contain a FAD binding signature (G(A,G,S,TGX(A,G,S,T)G) and an adenosine binding signature (GXGXXG) (Rossmann fold motif) (Fraaije *et al.*, 2002)

Following a method developed by Van Beilen and co-workers (Van Beilen *et* al., 2003) who designed highly degenerate primer sequences basing the forward sequence on a conserved region in the FAD binding Rossmann fold and the reverse sequence based on a NADPH binding Rossmann fold identified from the organisms *Acinetobacter* sp. NCIMB 9871 BVMO, *Rhodococcus Rhodochrous* IFO3338, steroid monooxygenase and putative BVMO sequence from *Pseudomonas fluorescens* DSM 50106 (discussed further in appendix I).

Experiments were carried out using these degenerate primer sequences. However, due to the nature of the work involved no sequences were isolated. This approach was fraught with problems, for example, extracting the DNA from the fungal mass proved difficult and required may attempts at breaking open the tough protoplasts under a variety of conditions. Optimisation of the PCR conditions was time consuming

requiring many different cycles to be attempted along with the use of different polymerases and reaction conditions. The outcomes of these initial experiments were disappointing and often lead to non-specific primer binding.
Despite these initial results this method provides a powerful tool and displays huge potential in the isolation of the CeBVMO from the fungus.

Due to the nature of the work involved this second method was not chosen as the main focus of the project. Had this route been chosen then further understanding on the fungal growth characteristics and investigation of other pathway enzymes would have been lost.

3.4 Summary

In this chapter successful growth of *C. echinulata* on a large scale was achieved. In order to process large quantities of material a suitable extraction technique using mechanical homogenisation has been established and used with success.

It has been shown that *C. echinulata* is NADPH dependent and therefore may be classed as a Type 1 monooxygenase, similar to that of most other known monooxygenase systems, as discussed previously in section 1.3.3.

A lactone hydrolase enzyme has been found in the *C. echinulata* BVMO system, similar to that found in the *A. calcoaceticus* CHMO system, known to act upon the lactones produced. It was therefore necessary to inhibit the lactone hydrolase activity during the bioconversion reactions in order to track CeBVMO activity throughout the purification procedure. It was found that Alloxan and E-64 were the best candidates with Alloxan being the inhibitor of choice throughout the extraction process.

Protein purification was carried out with success to an extent, resulting in two purification steps used, hydrophobic interaction followed by Reactive Red NADPH interaction chromatography resulting in a possible CeBVMO protein location at 50KDa-70KDa. However, when visualising the proteins all gels required silver staining due to the low quantities of protein present. This method of staining brings about several problems, the first of which is the amount of protein needed to carry out N-terminus sequencing. Ideally milligrams of material are required. In this case nanograms of proteins were isolated. Secondly, N-terminus sequencing cannot be carried out on proteins that have been silver stained. These two issues were the fundamental problems faced during the isolation and purification of the enzyme.

CHAPTER 4

Isolation and purification of a lactone hydrolase

4.1 Introduction

In this chapter, the isolation of a hydrolase enzyme will be examined. This hydrolase enzyme has been shown to target the products of the BVMO reaction and could ultimately play an important role in a scaleable chemical process as part of a multi step biocatalytic reaction.

From the known biotransformation reactions about 90% of these fall in to the hydrolytic biotransformation category. These reactions involve enzymes such as proteases, esterases or lipases. These hydrolase enzymes cleave the covalent bonds using water molecules therefore having applications in cleavage of epoxides, nitriles and phosphate esters. (Loughlin *et al.*, 2000). The reactions are often chemoselective and stereoselective which is a huge benefit for chiral synthesis.

Some hydrolase reactions involve three residues which make up a catalytic triad. The first step of the reaction involves the attack of a nucleophilic group from the active site and the carbonyl group of the substrate. This nucleophile can either be a hydroxyl group of a serine residue (pig liver esterase), a carboxyl group of an aspartic acid (pepsin) or the thiol group of a cysteine (papain).

4.2 Hydrolase enzymes

4.2.1 Esterases

Esterases show the ability to catalyse the cleavage and formation of ester bonds and are widely found in plants, animals and microorganisms. Currently the most commonly used esterases are those isolated from porcine or equine sources. These have the ability to act on non-natural esters which makes them an excellent candidate for biotransformations in synthetic chemistry. The catalytic triad consists of serine, histidine and aspartic acid residues. The serine residue performs the nucleophilic attack on the substrate, figure 4.1.

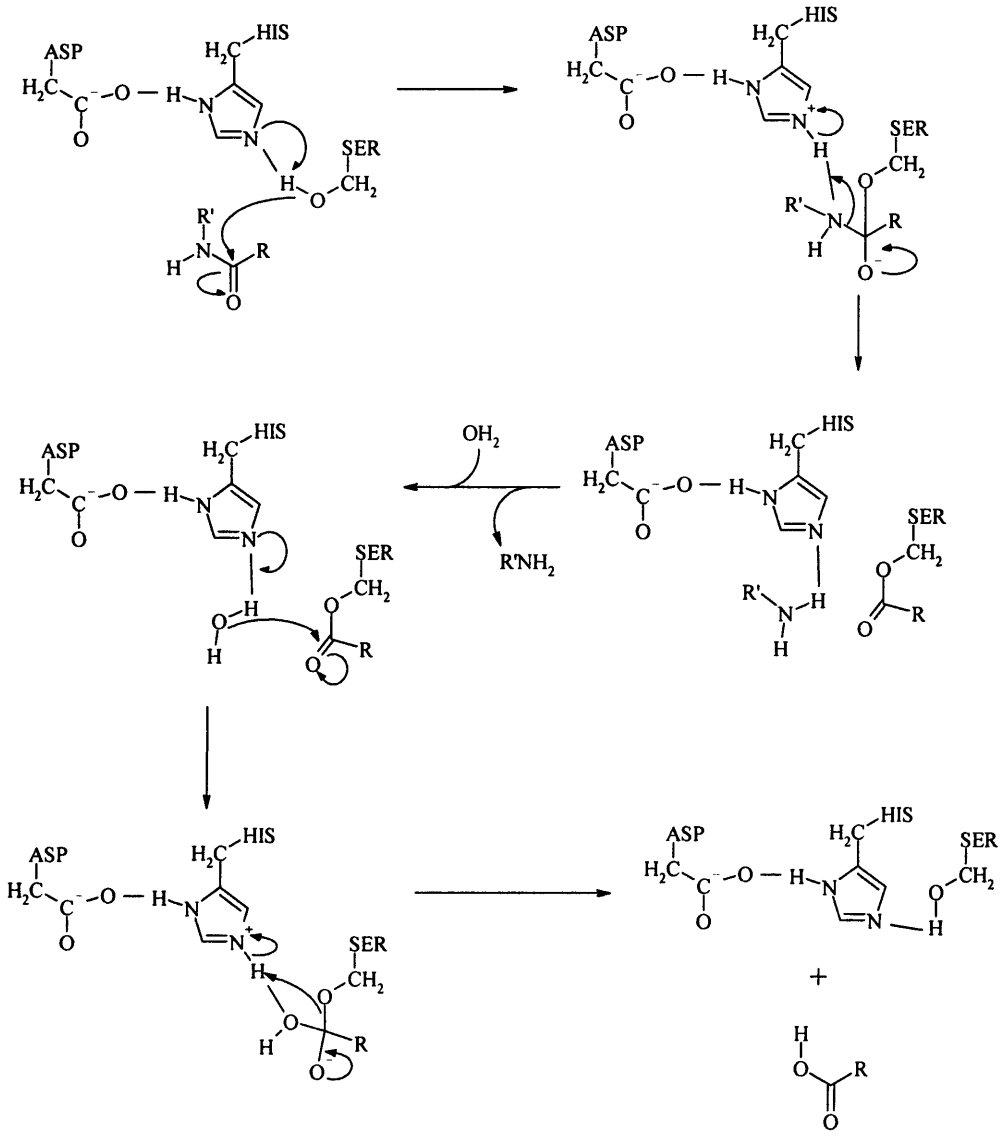


Figure 4.1: Mechanism of a serine based esterase

The carbonyl bond must first be polarised. This is achieved by hydrogen bonding to the carbonyl oxygen. Hydrogens are donated from NH groups on the backbone or from side chain amides (Asn or Gln). The carbonyl carbon becomes more electropositive and therefore more susceptible to nucleophilic attack.

The histidine group acts as a base and deprotonates the hydroxyl group of the serine thus making it more nucleophilic. Nucleophilic attack is fast and a tetrahedral intermediate is formed. This intermediate will not break down easily as there is no good leaving group present. In order to overcome this protonation occurs using the available imidazolium ion on the His side chain. The formation of an amino group provides a suitable leaving group leading to the breakdown of the tetrahedral species in to an ester intermediate. This ester intermediate species must be broken down further via nucleophilic attack on the newly formed carbonyl group. Water is deprotonated by the deprotonated His imidazolium forming a nucleophile which attacks the carbonyl group. The resulting intermediate species is protonated using the re-protonated His which acts as a general acid creating a ser-oxygen leaving group leaving the resulting product.

4.2.2 Lactone hydrolases

Lactones are essential in industry as fragrance or flavour components in foods, in nature as sex pheromones and also form the basic building block of more complex macromolecules.

Current synthetic methods rely on the Baeyer-Villiger reaction. However, regioselectivity cannot be controlled and often the final product is a mixture of lactones which proves a problem within the pharmaceutical industry. Lactone hydrolase enzymes provide one such method for separating out racemic lactones, by hydrolysis to form hydroxy-carboxylic acids. Research in to this area has yielded many stereospecific lactonases, and it has become clearer that they show the ability to create chiral compounds from pro-chiral carbonyl compounds. For example, the production of **D**-pantolactone, a chiral building block for **D**-pantothenic acid. Current synthetic methods to produce pantolactone relies on an expensive alkaloid to resolve the racemic **D**-pantolactone. However, a lactone hydrolase isolated from *Fusarium oxysporum* AKU3702 shows the ability to resolve the **D**-pantolactone providing an inexpensive method compared to that of the current synthetic process. Interestingly, a lactone

hydrolase isolated from *Agrobacterium tumefaciens* has been shown to resolve the pantolactone to the opposite enantiomer. (Shimizu, 2001; figure 4.2).

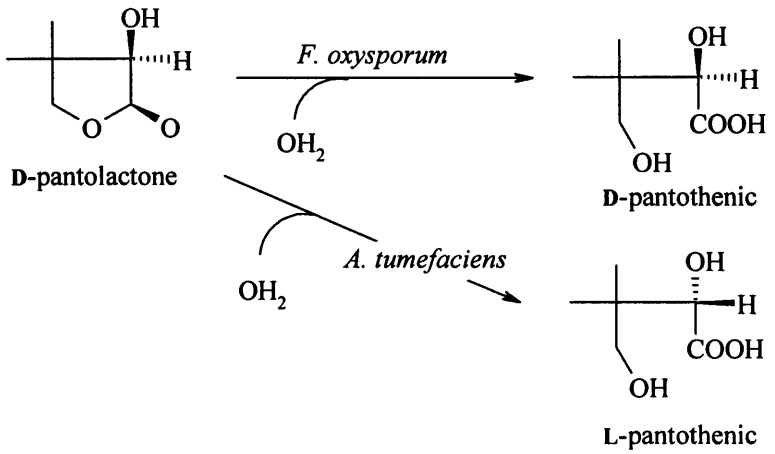


Figure: 4.2: Conversion of D-pantolactone to D-pantothenic and L-pantothenic acid using *Fusarium oxysporum* AKU3702 and *Agrobacterium tumefaciens*.

Lactone hydrolases, although capable of performing enantiospecific biotransformations are not widely used. Screen of their potential sources, their biochemistry, structures and functions have not been sufficiently studied in detail. This has made them difficult to identify and requires more research before they can be considered as a viable alternative for the traditional organic synthetic methods.

In this chapter function of the lactone hydrolase from *C. echinulata* is investigated with respect to the specific action on the lactones produced from the bioconversion of (+/-) bicyclo[3.2.0]hept-2-en-6-one.

4.3 Materials and Methods

Unless stated otherwise all chemicals were of the highest purity available from Sigma-Aldrich Chemical Company (Poole, Dorset, U.K.) Growth media components were obtained from Oxoid Ltd (Basingstoke, Hants, U.K.) *Cunninghamella echinulata* NRRL 3655 was obtained from LGC (Teddington, Middlesex, U.K.).

4.3.1 Inoculum

As section 2.3.7.1

4.3.2 15L Fermentation

As section 2.3.7.2

4.3.3 Lactone Hydrolase activity assay

The general esterase substrate, *para*-nitrophenyl acetate (pNPHOAc) was used to monitor the lactonase activity where the conversion of the colourless pNPHOAc to a yellow solution of *para*-nitrophenyl can be followed. pNPHOAc was prepared as a 10 mM stock in ethanol. All readings were taken at 348 nm over one minute. In a typical procedure cell extract (100 µl) was added to 100mM sodium acetate buffer (pH 6, 800 µl) and pNPHOAc (100 µl).

4.3.4 Isolation of the Lactone Hydrolase enzyme from the fungal mass

4.3.4.1 Method one:- from submerged culture

The fungal pellets from the fermentation were harvested by filtration through a Miracloth membrane (20-22 μm pore size) (Calbiochem, Notts, U.K.) and washed with 1.5 L of 100 mM sodium phosphate buffer (pH 8). The fungal pellets were then re-suspended into 3 L of 100mM sodium phosphate buffer (pH 8). The fungal mass was then homogenised using a high pressure LAB 60 APV-Gaulin homogeniser (APV-Gaulin, Crawley, Sussex, UK) at one pass at 0 bar followed by two passes at 200 bar. The homogenised fungal mass was then centrifuged (5000g, 30 mins, 4°C) and the supernatant (approx 4 L) separated from the deposit and concentrated by tangential flow using 3 × 50 cm^2 10KDa cut off tangential concentrators (Millipore, Watford, UK).

4.3.4.2 Method two:- from shake flask culture

Fungal biomass from shake flasks fermentations was removed by filtration and washed with 100 mM sodium phosphate buffer (pH 8). 5 g of fungal biomass was added to 10 ml 100 mM sodium acetate buffer (pH 6) and sonicated at 18 μ on ice, eight times for 30 sec periods with one minute rest between each sonication step. The suspension was then centrifuged (10,000g, 30 min, 4 °C) and the supernatant decanted from the deposit. The supernatant was then concentrated using a centrifugal concentrator, 10KDa cut off (Vivascience Ltd, Epsom, U.K.).

4.3.5 Lactone hydrolase inhibition

Fungal biomass was cultured as before (section 4.2.1) and protein extracted by sonication .

Alloxan was prepared at 10 mM, E64 was prepared at 14 μM/ml and AEBSF at 1mM. The general esterase substrate, *para*-nitrophenyl acetate (pNPHOAc) was used to monitor the lactone hydrolayse activity. pNPHOAc was prepared as a 10mM stock in ethanol. All reading were taken at 348 nm (UV2 AT-UVicam, Uvicam Ltd, Cambs, UK) as this represents the isobestic point, the wavelength at which the absorbance is pH independent. Reactions were set up as detailed below

Substrate blank

100 µl +10KDa cell extract was added to 800 µl 100 mM sodium acetate buffer (pH 6) followed by the addition of 100 µl ethanol (in place of the pNPHOAc)

Inhibitor assay

100 µl +10KDa cell extract was added to 800 µl 100mM sodium acetate buffer (pH 6) followed by the addition of 100 µl inhibitor and left at room temperature for 30mins after which 100 µl pNPHOAc was added and absorbance readings taken.

4.3.6 Ion Exchange Chromatography Columns (IEX)

Vivapure Mini H starter (Vivascience Ltd, Epsom, U.K.) was used containing four classes of ion exchange columns.

Sulphonic acid (S)	strong acidic cation exchanger	$R\text{-}CH_2\text{-}SO_3^- Na^+$
Carboxylic acid (C)	weak acidic cation echanger	$R\text{-}COO^-$
Quarternary ammonium (Q)	strong basic anion exchanger	$R\text{-}CH_2\text{-}N^+\text{-}(CH_3)_3Cl^-$
Diethylamine (D)	weak basic anion exchanger	$R\text{-}CH_2\text{-}N\text{-}(C_2H_5)_2$

4.3.6.1 Quaternary ammonium and diethylamine columns

5 ml of sonicated material in 100 mM sodium acetate buffer (pH 6) (as described in section 4 .3.4.2) was buffer exchanged against loading buffer (25 mM Tris HCL (pH 8)) The columns (Q and D) were equilibrated with 400 µl loading buffer and centrifuged (13000 rpm, 5 mins, 4°C).

400 µl of sample was loaded on to the spin column and centrifuged (13000 rpm, 4°C, 5 mins) followed by two further washes of loading buffer (400 µl) and centrifugation. The protein was eluted from the column using 400 µl 1.5M NaCl dissolved in 25 mM Tris HCl pH 8.

4.3.6.2 Sulphonic acid and carboxylic acid columns

5 ml of sonicated material in 100 mM sodium acetate buffer (pH 6) (as described in section 4.3.4.2) was buffer exchanged against loading buffer (25 mM sodium acetate (pH 5.5))

The columns (S and C) were equilibrated with 400 µl loading buffer and centrifuged (13000 rpm, 5 mins, 4°C).

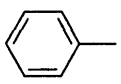
400 µl of sample was loaded on to the spin column and centrifuged (13000 rpm, 4°C, 5 mins) followed by two further washes of loading buffer (400 µl) and centrifugation. The protein was eluted from the column using 400 µl 1.5 M NaCl dissolved in 25 mM sodium acetate pH 5.5.

4.3.6.3 Optimisation of protein elution from quaternary ammonium column

5 ml of sonicated material in 100 mM sodium acetate buffer (pH 6) (as described in section 4.3.4.2) was buffer exchanged against loading buffer (25 mM Tris HCL (pH 8)). 5 ml of buffer exchanged material was loaded directly on to a 20ml Q anion exchange column (Vivascience Ltd, Epsom, U.K). Proteins were eluted by a series of high salt buffers ranging from 10 mM to 1.5M NaCl dissolved in loading buffer (25 mM Tris HCl (pH 8)). All fractions were collected and assayed for lactone hydrolase activity. The active fractions were collected and concentrated though a 10KDa cut off centrifugal concentrator (4000 rpm, 30 min, 4 °C) (Vivascience Ltd, Epsom, U.K.).

4.3.7 HiTrap Hydrophobic Interaction Chromatography Columns (HIC)

HiTrap 1ml column selection kit was obtained from Amersham Pharmacia Biotech.

Phenyl Speharose high performance	Highest ionic strength	
Octyl Sepharose		CH_3-$(CH_2)_6$-CH_2-
Butyl Sepharose	Lowest ionic strength	CH_3- CH_2- CH_2- CH_2- CH_2-

Buffers used:-	High Salt	100 mM Tris HCl (pH 8), 1M Ammonium Sulfate
	Low Salt	100 mM Tris HCl (pH 8)

The column was equilibrated with high salt buffer (10 ml). A 0.5 ml protein sample from the Q anion exchange active fraction was then loaded on to the column followed by a high salt wash (10 ml) and low salt wash (10 ml). Fractions were then collected from each wash and concentrated though a 10KDa cut off centrifugal concentrator (4000 rpm, 30 min, 4 °C) (Vivascience Ltd) and run on a 19.5% SDS-PAGE gel and analysed for lactonase activity.

4.3.8 Gel filtration

HiTrap Sephadex G25 superfine 5 ml column was obtained from Amersham Pharmacia Biotech.

5 ml of high salt elute from Octyl Sepharose was dialysed (MWCO 8KDa, Medicell International Ltd, London, UK) for 24 hours with 3 changes of 100 mM Tris HCl buffer (pH 8). The column was equilibrated with low salt buffer (Tris HCL,100 mM, pH 8, 10 ml) at 4°C. A 0.5ml protein sample was loaded on to the column followed by a high salt wash (100 mM Tris HCl (pH 8), 0.75M NaCl, (5 ml)). 100 µl fractions were collected and assayed for lactonase activity.

4.3.9 Effect of lactone hydrolase on lactones

1 ml samples of supernatant from a previous whole cell bioconversion know to contain the enzyme induced lactone (3-oxabicyclo[3.3.]oct-6-en-2-one: Previously described in section 3.2.4) and 2-oxabicyclo[3.3.0]oct-6-en-3-one (1 mg/ml) were incubated with 100 µl of high salt cell extract from Q anion exchange in a reciprocating shaker at 170 rpm, 28°C for 24 hours. Samples were then analysed by GC as described previously in section 3.2.5.

4.4 Results

4.4.1 Isolation and purification of the lactone hydrolase

As previously discussed in section 3.3.5 the lactone hydrolase present within the fungus showed the ability to cleave the lactones produced from the bioconversion of (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone. This hydrolase enzyme was initially found during cell free experiments using material that had been cultured for eight days (pH of 8-8.5) displaying BVMO activity. However, it has been found that lactone hydrolase activity can be monitored in the fungus after 24 hours growth during shake flask culture therefore eliminating the requirement for running a 15L fermentation for eight days.

It must be noted the standard activity test employed to monitor the hydrolase activity is one that is routinely used to monitor esterase enzyme activity and is therefore not a specific test for the suggested lactone hydrolase present in *C. echinulata.* It is however a useful and rapid test to monitor the presence of an esterase type enzyme when performing initial protein isolation.

A specific assay was developed in order to monitor the activity of the lactone hydrolase on the two lactones, 3-oxabicyclo[3.3.0]oct-6-en-2-one and 2-oxabicyclo[3.3.0]oct-6-en-3-one which encompassed the use of a colourimetric screen in combination with GC analysis.

4.4.1.1 Ion Exchange Chromatography

Ion exchange chromatography columns were used in the first instance as an initial purification step.

It was found that the lactone hydrolase bound to Q and weakly to D (basic anion exchanger) but no binding occurred using S and C (acidic cation exchanger).

The elution process can be taken one step further by eluting proteins from the column using a stepwise gradient of NaCl. Using this gradient protein can be separated on the basis of charge and size. This elution method ensures that the protein of interest is removed with the least number of contaminating proteins.

Table 4.1 shows the lactone hydrolase activity in each fraction of a stepwise elution from a 1ml Mini Q spin column.

Table 4.1: lactone hydrolase activity from each fraction eluted from a Mini Q ion exchange column.

Q Column Fraction	Activity µmol/min/ml
Crude Extract	77.89
Flow through	4.6
10mM NaCl	6.42
20mM NaCl	19.47
30mM NaCl	1.26
40mM NaCl	7.59
50mM NaCl	2.14
1.0M NaCl	1.94

It can be seen that the highest activity was isolated in the 20mM NaCl elution although substantial loss in activity was observed compared to the activity in the crude extract.

The experiment was repeated using a 20ml Maxi Q column with a protein binding efficiency of 60-80mg eluting with at 20mM NaCl, table 4.2.

Table 4.2: lactone hydrolase activity from each fraction eluted from a Maxi Q ion exchange column

Q Column Fraction	Activity µmol/min/ml
Crude Extract	38.56
Flow through	3.31
20mM NaCl	26.09
1.0M NaCl	6.62

Using the Maxi column the highest activity was again observed in the 20mM NaCl elution. SDS PAGE gels were run of all eluted fractions from the Mini and Maxi columns. Below in figure 4.3 a wide range of protein separation can be seen when using a stepped elution gradient. However, in figure 4.4 clear protein bands can be seen in the active 20mM NaCl elution with proteins clearly seen in the sub 25KDa range.

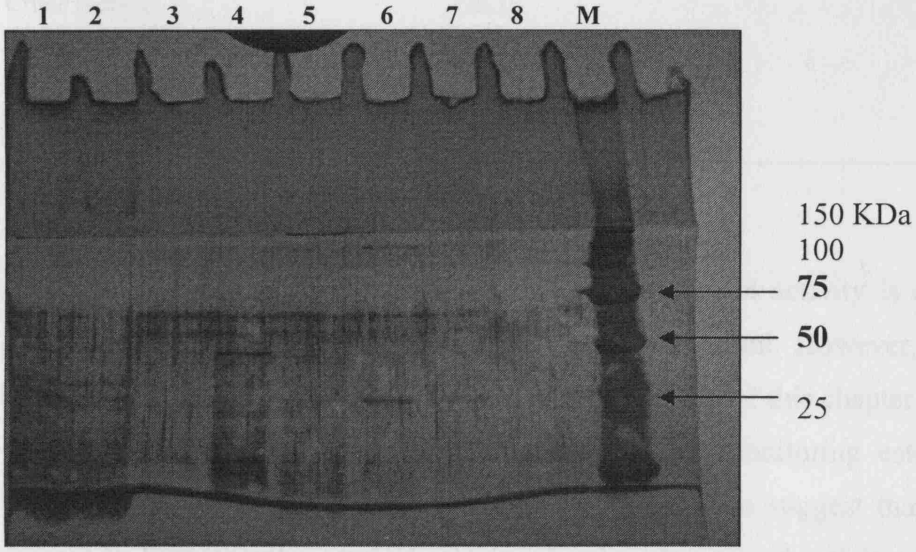


Figure 4.3: Silver stained SDS PAGE gel protein elutions from a mini Q ion exchange column. 1- crude protein 2- flow through 3-wash 4-10mM NaCl 5-20mM NaCl 6-30mM NaCl 7-40mM NaCl 8-50mM NaCl M-markers

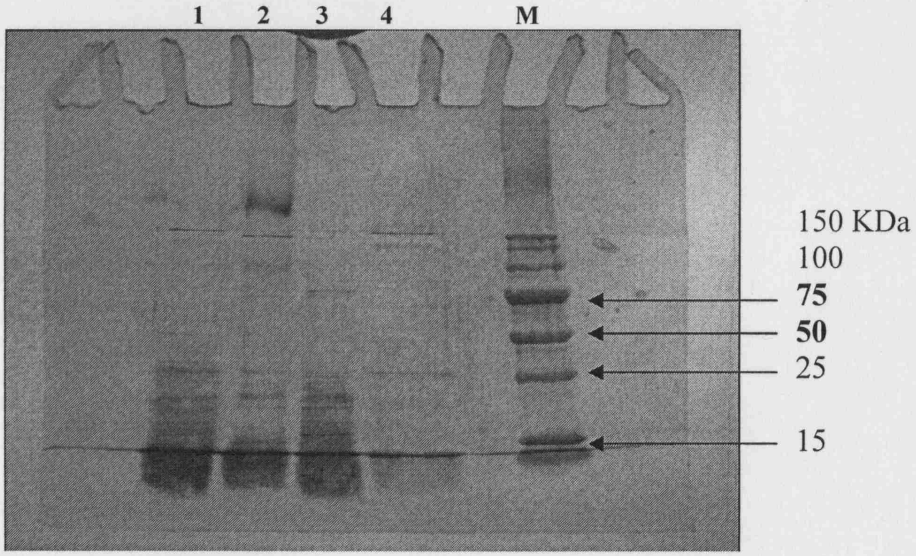


Figure 4.4: Silver stained SDS PAGE gel protein elutions from a Maxi Q ion exchange column. 1- crude protein 2- flow through 3-20mM NaCl 4-1M NaCl M-markers

In order to further purify out the lactone hydrolase from the active fraction, the 20mM elution was spun though a 30KDa spin concentrator and the two resulting fractions assayed for activity, table 4.3.

Table 4.3: lactone hydrolase activity from each fraction eluted from a Maxi Q ion exchange column followed by concentration through a 30KDa spin concentrator.

Fraction	Activity µmol/min/ml
Crude Extract	120.16
Q- 20mM NaCl	88.02
Q- 1M NaCl	31.54
10-30KDa	49.85
+30KDa	123

From the data in table 4.3 it can be seen that the highest activity is observed in the +30KDa fraction with an activity rate of 123 µmol/min/ml. However, the 10-30KDa fraction still shows high activity. As discussed at the start of this chapter the activity test used to monitor the reaction is a standard test for monitoring esterase enzymes. Therefore observing activity in both fractions is likely to suggest that an esterase is present in both samples, each displaying varying degrees of activity. These fractions were subjected to a specific lactone hydrolase test which is discussed further in section 4.4.2.

4.4.1.2 Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography columns (HIC) were investigated as a second purification step. In this experiment (table 4.4) bound protein was eluted from the Q column at a 1M NaCl concentration. The activity recorded was taken from a 5ml fraction collection and not concentrated which takes in to account for the lower activity compared to that of the crude extract. Out of the three HIC columns tested highest activity was observed in the high salt fractions, indicating that a majority of proteins had not bound, or possibly due to column overloading. However, octyl and phenyl sepharose did show some activity (~ 22-26 μmol/min/ml) in the low salt elution, i.e. from proteins that had bound to the column.

Table 4.4: Lactone hydrolase activity using hydrophobic interaction chromatography columns.

Column	Fraction	Activity μ/mol/min/ml
Crude Extract	Crude	77
Q Anion Exchange	High Salt	35.8
Q Anion Exchange	Low Salt	8
Butyl Sepharose	High Salt	55
Butyl Sepharose	Low Ssalt	6.6
Octyl Sepharose	High Salt	78
Octyl Sepharose	Low Salt	22
Phenyl Sepahrose	High Salt	39
Phenyl Sepahrose	Low Salt	26

As previously mentioned, the use of the octyl sepharose column gave rise to activity in both fractions, highest located in the high salt elution. A further experiment was performed to ascertain if this column had separated two distinct esterase type enzymes. Table 4.5 shows the results after applying a 20mM NaCl Q column elution to an octyl sepharose HIC column. The results show that almost equal activity is split between high and low salt elutions from the octyl sepharose column. Gel filtration was used on the low salt elution from octyl sepharose but due to very low protein concentrations gel visualisation using silver stain did not prove to be satisfactory.

Table 4.5: Lactone hydrolase activity using IEX and HIC columns combined.

Column	Fraction	Activity µmol/min/ml
Crude extract	Crude	113.92
Q Anion Exchange	Low Salt	12.85
Q Anion Exchange	20mM NaCl	30.18
Q Anion Exchange	1M NaCl	0.77
10KDa Concentrator	20mM NaCl concentrated 15 fold	110.03
Octyl Sepharose	High Salt	52.58
Octyl Sepharose	Low Salt	57.25

As previously discussed in chapter three this lactone hydrolase can be inhibited by the addition of Alloxan and E-64. Both of these inhibitors affect cysteine based enzymes, Alloxan inhibits thiol groups by oxidation and E-64 is an irreversible and highly selective cysteine protease inhibitor. It can therefore be concluded that the hydrolase of interest is cysteine based. With time permitting the next step in the purification process would have been to attempt to isolate the hydrolase using affinity chromatography. The use of thiol sepharose 4B (Amersham Biosciences) would have allowed the separation of this thiol containing protein.

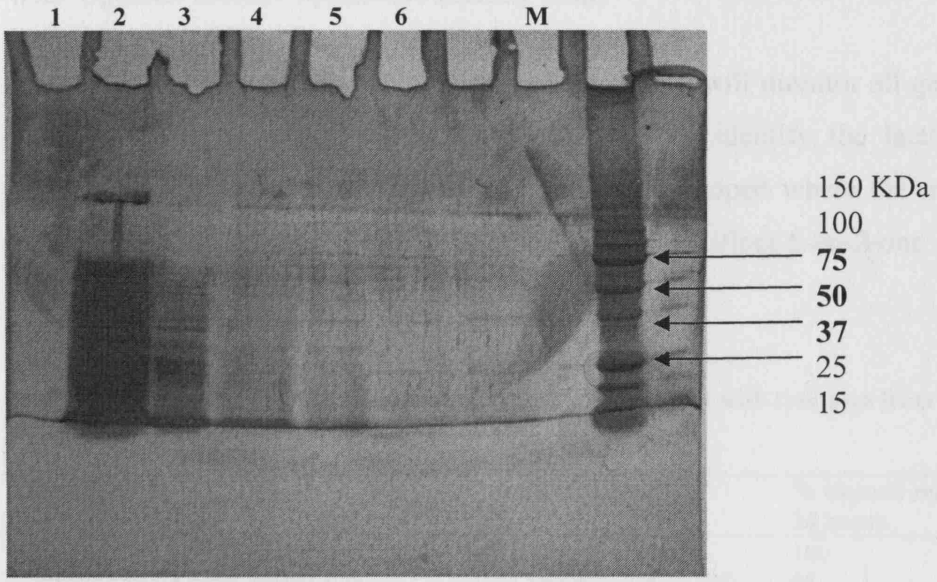


Figure 4.5: Silver stained SDS PAGE gel of proteins from Q and octyl sepharose columns
1- crude 2-Q Flow through (low salt) 3- 20mM NaCl 4-1M NaCl 5-Octyl HS 6-Octyl LS

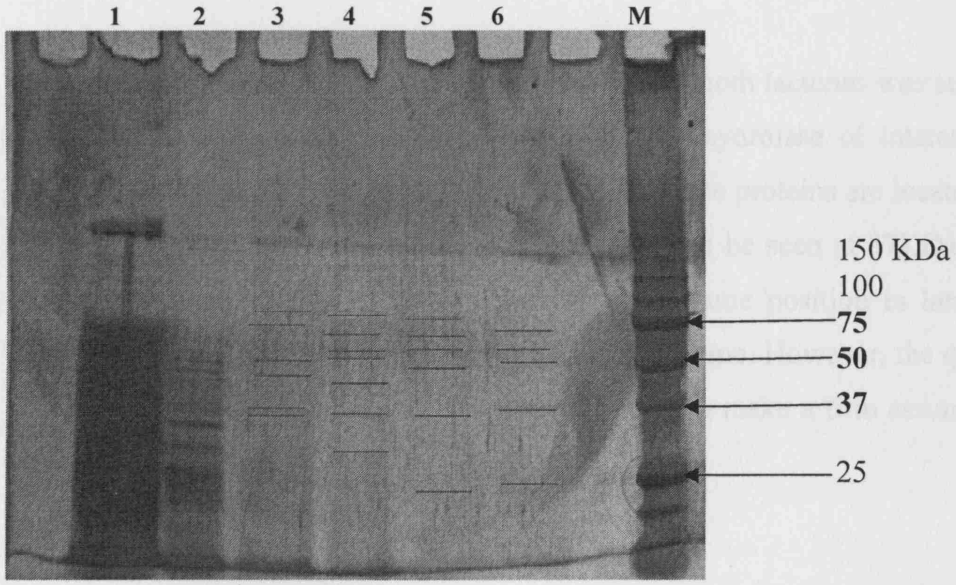


Figure 4.6: Enhanced silver stained SDS PAGE gel showing highlighted protein bands from Q and octyl sepharose columns
1- crude 2-Q Flow through (low salt) 3- 20mM NaCl 4-1M NaCl 5-Octyl HS 6-Octyl LS

4.4.2 Specific lactone hydrolase activity assay

As previously discussed the general nitrophenyl assay will monitor all general esterase active enzymes. In order to specifically isolate and identify the lactone hydrolase enzyme from *C. echinulata* a specific assay was developed where the reduction of 2-oxabicyclo[3.3.0]oct-6-en-3-one and 3-oxabicyclo[3.3.0]oct-6-en-2-one lactones was observed.

Table 4.6: Percentage lactone remaining after 24 hours reaction with fractions from combined purification using Q followed by octyl sepharose HIC column.

Lactone	Column fraction	% lactone remaining after 24 hours
2-oxabicyclo[3.3.0]oct-6-en-3-one	Buffer	100
2-oxabicyclo[3.3.0]oct-6-en-3-one	Octyl Sepharose High Salt	98
2-oxabicyclo[3.3.0]oct-6-en-3-one	Octyl Sepharose Low Salt	17
3-oxabicyclo[3.3.0]oct-6-en-2-one	Buffer	100
3-oxabicyclo[3.3.0]oct-6-en-2-one	Octyl Sepharose High Salt	72
3-oxabicyclo[3.3.0]oct-6-en-2-one	Octyl Sepharose Low Salt	13

It was observed (table 4.6) that the biggest decrease of both lactones was seen using the low salt elution fraction suggesting that the lactone hydrolase of interest is located within this fraction. Referring back to figure 4.5, possible proteins are located at 37KDa and 50-75KDa ranges. However, a prominent band can be seen at 37KDa in lane six, only a very weak protein signal is observed at the same position in lane five. It is therefore possible that this is the lactone hydrolase enzyme. However, the quality of the gel and amount of protein present is not high enough to make a firm assumption based on these results alone.

The fractions isolated from Q IEX (table 4.3) were also subjected to the same assay and the results are shown in table 4.7 below.

Table 4.7: Percentage lactone remaining after two hours reaction with 20mM NaCl fraction eluted from Q IEX and subjected to size separation.

Lactone	Lactone hydrolase fraction	% lactone remaining after 2 hours
3-oxabicyclo[3.3.0]oct-6-en-2-one	-10KDa	36
3-oxabicyclo[3.3.0]oct-6-en-2-one	10-30KDa	47
3-oxabicyclo[3.3.0]oct-6-en-2-one	+30KDa	44
2-oxabicyclo[3.3.0]oct-6-en-3-one	-10KDa	30
2-oxabicyclo[3.3.0]oct-6-en-3-one	10-30KDa	29
2-oxabicyclo[3.3.0]oct-6-en-3-one	+30KDa	27

From the data above no clear conclusion can be drawn from the individual fraction sizes isolated. It is possible that proteins of a higher or lower molecular weight have passed through or held back during concentration though the spin columns therefore creating impure protein fractions. However, what can be concluded is that like *A. calcoaceticus*, which is known to contain a hydrolase enzyme as part of the full enzyme cycle cleaving the lactones, *C. echinulata* also contains a lactone specific hydrolase enzyme which has been demonstrated from this set of experiments.

4.4.3 Shotgun cloning of lactone hydrolase

In order to approach the isolation of this lactone hydrolase using different methods an attempt at shotgun cloning was performed. Although this method carries a low success rate it is possible that a hit could be made against the hydrolase enzyme as these enzymes are often small non-complex proteins encoded by small DNA fragments, therefore the possibility of obtaining a positive hit is slightly increased. The use of the rapid visual nitrophenyl screen enabled this approach to be considered as multi-well plates could be assayed in several minutes. From the library generated three possible clones were identified. Unfortunately, these clones have not been sequenced due to the generation of the library in the last stages of this project (discussed further in appendix II).

4.5 Summary

From the experimental data a lactone hydrolase has been found in the fungus *C. echinulata.* It has long been known that a similar enzyme operates in *A. calcoaceticus* where the cleavage of the lactone ring system results in an hydroxy acid.
Based on this comparison it would be feasible to assume that the same occurs in *C. echinulata* and the lactone form is cleaved to its hydroxyl acid form. However, throughout the project GC analysis was used which detected ketones, alcohols and lactones only. To detect and fully followed the reaction path a HPLC assay would be the ideal choice which would allow detection of this hydroxy acid form. However, due to time constraints on the project developing a suitable assay for such analysis was not achievable.

As an initial isolation of this lactone hydrolase a basic purification protocol has been established using ion exchange in combination with hydrophobic interaction chromatography. Some of the main problems encountered frequently were the limited amounts of fungal material available. As previously stated, the discovery that the hydrolase was present after 48 hours growth was a major benefit to reducing the culture time. It must also be noted that each batch of fungal material varied in activity. This variation occurred in both material cultured for 24 hours and eight days and would vary ± 30%. This degree of variation was not investigated, however, no significant difference was observed in activity between 48 hours and eight days growth.

CHAPTER 5

Identification of an alcohol dehydrogenase

5.1 Introduction

This chapter describes the identification and characterisation of an alcohol dehydrogenase present in *C. echinulata* which has been shown to convert cyclic alcohols in to cyclic ketones.

Using the racemic starting substrate (+/-)bicyclo[3.2.0]hept-2-en-6-one ketone, two lactones are produced, 2-oxabicyclo[3.3.0]oct-6-en-3-one and 3-oxabicyclo[3.3.0]oct-6-en-2-one in enantiomerically pure forms. The BVMO produced by *C. echinulata* predominantly produces the 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone under whole cell biotransformation conditions (Alphand *et al.*, 2000). It is this unique property of selectivity that makes this fungal system novel compared to the cyclohexanone 1,2-monooxygenase isolated from *A. calcoaceticus* NCIMB 9871, that produces the lactones in an equal ratio. This AcCHMO system has been extensively studied (Alphand *et al.*, 1990a/b; Roberts *et al.*, 1993 and Taschner *et al.*, 1993) and can be induced during growth to metabolise cyclohexanol to adipate via five steps (Donoghue and Trudgill, 1975). The conversion of cyclohexanol to cyclohexanone in the *A. calcoaceticus* pathway is a coupled enzyme system that uses an alcohol dehydrogenase and Baeyer-Villiger monooxygenase in a closed loop recycling procedure (Willetts, 1997), figure 5.1. This system is an important attribute of the AcCHMO pathway and would be assumed that it is also present in the fungal CeBVMO system. Its presence in *C. echinulata* would provide a new step in the conversion of bicyclo[3.2.0]hept-2-en-6-one ketone to the respective lactones.

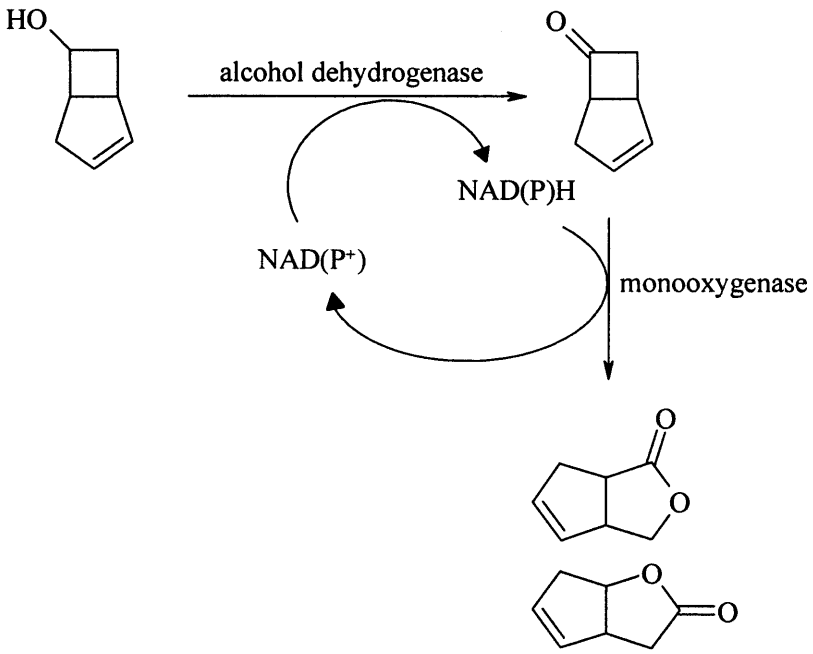


Figure 5.1: Reaction scheme showing the coupled alcohol dehydrogenase and Baeyer-Villiger monooxygenase enzyme system.

5.2 Materials and Methods

Unless otherwise stated all chemicals were of the highest purity available from Sigma-Aldrich Chemical Company (Poole, Dorset, UK) Growth media components were obtained from Oxoid Ltd (Basingstoke, Hants, U.K.) *Cunninghamella echinulata* NRRL 3655 was obtained from LGC (Teddington, Midds, UK).

5.2.1 Analytical Gas Chromatography (G.C.)

All metabolites were identified by comparison of their retention times using an XL-2 gas chromatograph with flame ionisation detector (Perkin-Elmer, Norwalk, CT, USA) fitted with a ZB1 non-polar dimethylsiloxane column (30 m × 0.25 mm × 0.25 μm) (Phenomenex, Macclesfield, Cheshire, U.K.) with helium as the mobile phase with those of authentic compounds synthesised by classical chemical reactions or commercially available. 400 μl supernatant from the biotransformation was extracted with an equal volume of ethyl acetate containing naphthalene as the internal standard. Standard GC operating temperatures were set as follows: column 110°C, injector 250°C and detector 250°C. Chiral analysis was performed by GC using a Chiraldex-B column (25 m × 0.25 mm × 0.25 μm) (SGE, Milton Keynes U.K.) with helium as the mobile phase with operating temperatures as follows: column 90°C 4mins followed by ramp at 15°C min to 120°C, injector 200°C and detector 200°C.

5.2.2 Fermentations

Fermentations were carried out using corn steep liquor media (CSL): CSL (20 g/L), glucose (4 g/L), KH_2PO_4 (1 g/L), K_2HPO_4 (2 g/L), $NaNO_3$ (2 g/L), KCl (0.5 g/L), $MgSO_4$ (0.5 g/L), $FeSO_4$ (0.02 g/L).

Fermentations were performed in 250 ml non baffled shake flasks containing 100 ml CSL which were inoculated using fungal spores taken from a CSL slope and incubated at 28°C, 200 rpm over a period of 24 hours to seven days.

5.2.3 pH activity assay

In a typical procedure the fungal pellets were harvested by filtration through a Miracloth membrane (20-22 μm pore size) (Calbiochem, Notts, UK). Two sets of reactions were set up. The first using fungal biomass cultured over a 24 hour period, the second using fungal biomass cultured over a seven day period. Reactions were set up over a range of pH5-10 (listed below) using 5 g of cells which were re-suspended into 50 ml of pH buffer and incubated with 50μl bicyclo[3.2.0]hept-2-en-6-ol (1g/L) at 200 rpm, 28°C for 24 hours.

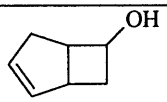
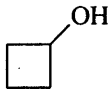
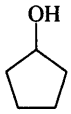
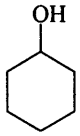
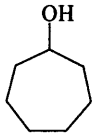
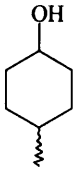
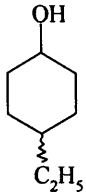
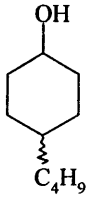
Buffers

pH 5 and pH 6	Sodium acetate 50 mM
pH 7 and pH 9	Tris HCl 50 mM
pH 10	Tris-Borate 50 mM

5.2.4 Substrate specificity

In a typical procedure the fungal pellets were harvested by filtration through a Miracloth membrane (20-22 μm pore size) (Calbiochem, Notts, UK) and washed with 100 ml of 100 mM phosphate buffer (pH 8). 5 g of cells were re-suspended into 50 ml 50 mM Tris HCL buffer (pH 7) and incubated with 50μl of the substrates shown in table 5.1 in a reciprocating shaker at 170 rpm, 28°C for 24 hours.

Table 5.1: Alcohol substrates used for substrate specificity of the alcohol dehydrogenase purchased from Sigma Aldrich

Alcohol substrates	
	Bicyclo[3.2.0]hept-2-en-6-ol
	Cyclobutanol
	Cyclopentanol
	Cyclohexanol
	Cycloheptanol
	(+/-) 4-methylcyclohexanol
	(+/-) 4-ethylcyclohexanol
	(+/-) 4-butylcyclohexanol

5.2.5 Synthesis of bicyclo[3.2.0]hept-2-en-6-ol

Bicyclo[3.2.0]hept-2-en-6-one (1 ml) was dissolved in ethanol (5 ml) with stirring. Sodium borohydride (0.75 g, 0.02 moles) was dissolved in water (1.5 ml) and added drop wise to the ketone mixture over 40 minutes with stirring. Water (10 ml) and concentrated HCl (1ml) was added to quench the reaction. The aqueous layer was washed using ethyl acetate (3 × 10 ml washes). Evaporation under reduced pressure yielded bicyclo[3.2.0]hept-2-en-6-ol (1 g) as a clear liquid (Netwon *et al.*, 1979).

The spectroscopic properties (^{1}H NMR) were consistent with the assigned structure according to literature (Giovannini *et al.*, 1996).

5.3 Results

5.3.1 pH activity assay

Figures 5.2 and 5.3 show the initial rate of reaction after 24 hours using fungal biomass cultured after 24 hours and seven day period. It can be seen that the reaction profiles in both follow the same trend with optimum activity observed around pH 7 followed by a decrease in activity at higher pH. Based on these initial experiments all substrate specificity reactions were performed at pH 7 using fungal mass grown for 24 hours.

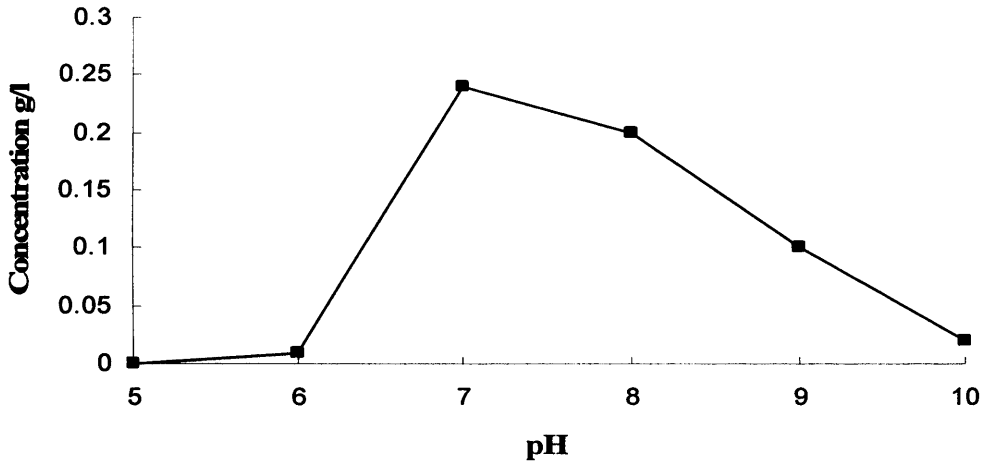


Figure 5.2: Ketone production after a 24 hour period using fungal biomass grown for 24 hours performed at different pH conditions.

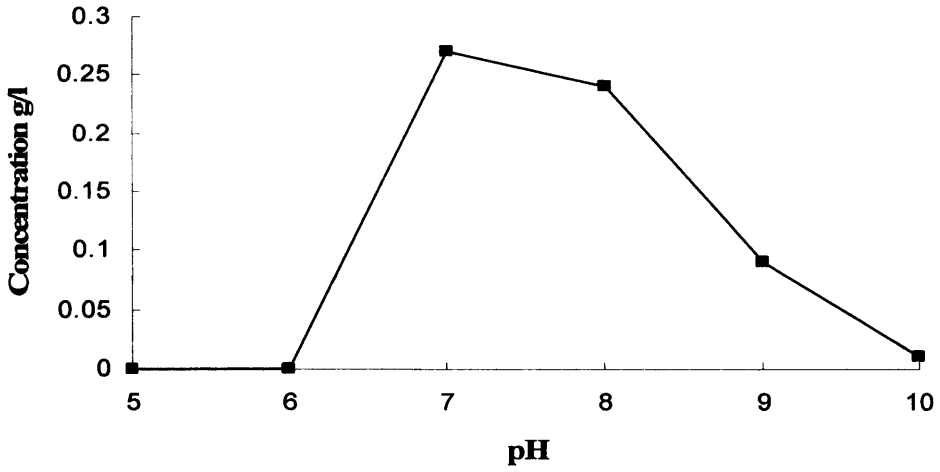


Figure 5.3: Ketone production after a 24 hour period using fungal biomass grown for seven days performed at different pH conditions.

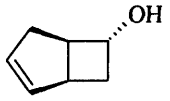
Performing pH activity assays carried out using cells known to display BVMO activity have shown that the optimum pH for the conversion of bicyclo[3.2.0]hept-2-en-6-ol to bicyclo[3.2.0]hept-2-en-6-one ketone is at pH 7. This observation is in direct comparison when growing *A. calcoaceticus* in the presence of ethanol as it has been shown by Abbott and co-workers that the conversion of ethanol to acetate had an optimum pH between range of 6.5 to 7.5 (Abbott *et al.*, 1973).

5.3.2 Substrate specificity

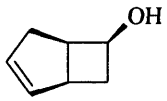
As previously discussed in chapter two *C. echinulata* was applied to a range of ketones in order to study specificity. Based on the same set of ketones used previously the corresponding alcohols were applied to *C. echinulata* for the dehydrogenase study.

Biotransformations were set up with *C. echinulata* using the selected alcohols. However, all but one of the alcohols used failed to yield the corresponding ketones.
From the seven alcohols used only cyclobutanone gave rise to its corresponding ketone (results not shown). Reactions were continued for a further five days after which a decrease in alcohol was observed in all samples, and unknown peaks were observed in the GC traces suggesting that the alcohols were undergoing breakdown in to products other than their expected ketones.

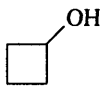
From this set of experiments it has been shown that *C. echinulata* readily converts bicyclo[3.2.0]hept-2-en-6-ol in to bicyclo[3.2.0]hept-2-en-6-one. It must be noted that classical chemical synthesis of the alcohol from ketone predominantly yields the *endo* form i.e. the less sterically strained form. This is discussed further in chapter 6 where chiral G.C analysis is performed. However, using the range of chosen alcohol substrates only cyclobutanol was converted in to cyclobutanone. Comparing the structures of bicyclo[3.2.0]hept-2-en-6-ol and cyclobutanol (figure 5.4) both contain a four ring cyclobutanone system. This ring system is naturally strained and therefore assumed that it will open readily. However, it is apparent that this small ring size is necessary in order to fit in to the active site of the alcohol dehydrogenase and that any other ring systems larger than a four membered ring will not undergo conversion.



exo bicyclo[3.2.0]hept-2-en-6-ol



endo bicyclo[3.2.0]hept-2-en-6-ol



cyclobutanol

Figure 5.4: Structures of *endo-* and *exo-* bicyclo[3.2.0]hept-2-en-6-ol and cyclobutanol

5.3.3 Reaction profile

From the previous set of experiment it has been shown that bicyclo[3.2.0]hept-2-en-6-ol can be successfully converted in to the (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone by the alcohol dehydrogenase present in *C. echinulata.* However, as the reaction was performed in whole cells after 24 hours growth, which from past experiments shows no BVMO activity, then it was assumed that the reaction would end after the alcohol conversion. Samples were taken from bioconversion reactions for six days and revealed the results shown.

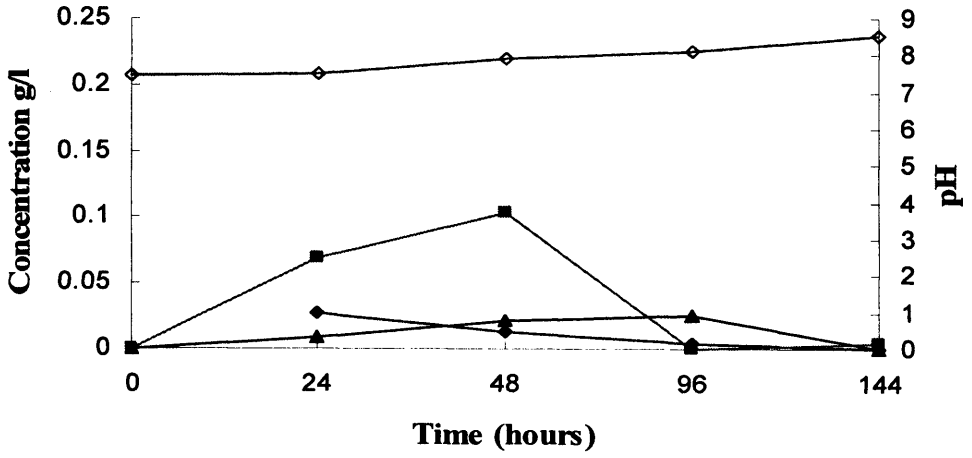


Figure 5.5: Bioconversion using fungal mass grown for seven days performed in media at pH 7.44. ♦- alcohol ■- ketone ▲- 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone ◊- pH

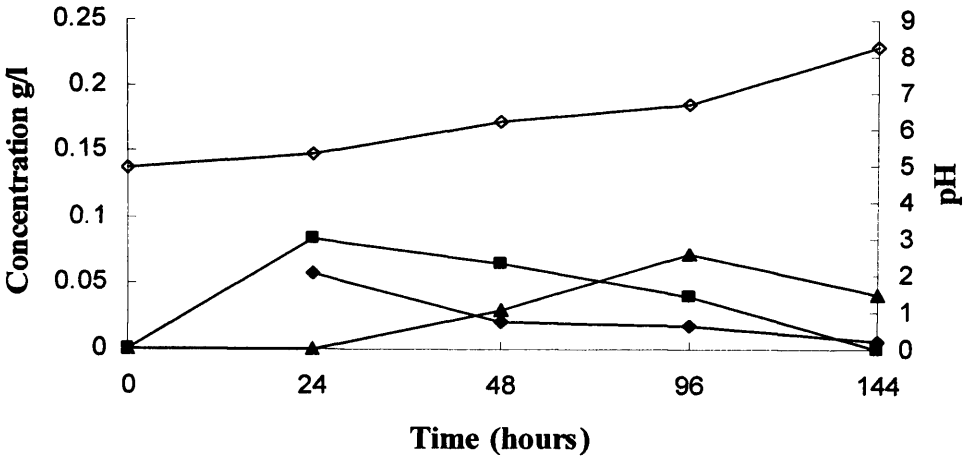


Figure 5.6: Bioconversion using fungal mass grown for 24 hours performed in media at pH 4.94. ♦- alcohol ■- ketone ▲- 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone ◊- pH

It can be seen in figures 5.5 and 5.6 that after 24 hours in both 7 day and 24 hour grown pellets both have produced similar amounts of ketone (0.06 and 0.08 g/l respectively). However, the ketone production in the 7 day old pellets continues to increase over the next 24 hour period resulting in 0.1 g/l decreasing from this point onwards whereas

ketone concentration decreases in 24 hour growth pellets and continues a steady decrease over the next 168 hours.

In both experiments alcohol consumption shows a steady rate of decrease with a consumption rate of 0.008g/l/h from 7 days growth and 0.0215g/l/h in 24 hours growth. It must be noted that the alcohol was added at a starting concentration of 1g/l. In both experiments after 24 hours only 0.05g/l is remaining. It is thought that this initial decrease is due to evaporation due to the volatility of the alcohol, if this were not the case then one would expect to see a large production of ketone after 24 hours which is not observed in the above experiments.

Lactone production in both experiments varies with the 24 hour grown pellets producing the highest amount of lactone (0.07 g/l) after 96 hours bioconversion. However, it is interesting to note that lactone production in 24 hour grown pellets is not observed until 48 hours growth where a change in pH from 5.3 to 6.2 (24 to 48hours) has occurred previously, whereas in the 7 day old pellets lactone production is observed after 24 hours where the pH of the medium is at pH 7.52. The pH in both experiments continues to increase steadily until > pH 8 is achieved in both after 144 hours.

It can be seen in both experiments that after 144 hours the lactone concentration has decreased. As a lactone hydrolase enzyme is known to exist in the whole cell system of *A. caloaceticus* then it is highly likely that what is observed in the whole cell system of *C. echinulata* does indeed follow a similar, if not the same degradation pathway as *A. calcoeceticus.*

As previously discussed in chapter two, the increase in pH observed during fermentation of *C. echinulata* plays a crucial role in the expression of the BVMO enzyme. It was observed that lactone was only synthesised after a pH rise had occurred, this was seen in both seven day and 24 hour shake flask growth. It is known from whole cell experiments in *A. calcoaceticus* that growth on cyclohexanol resulted in the degradation pathway as outlined in figure 5.1, and it has been shown by this set of experiments that *C. echinulata* also follows this same pathway with the conversion of alcohol → ketone → lactone.

An experiment was run (results not shown) using fungal mass grown over seven days (shake flask) at pH 7.44 adding (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone. Over a period of four days the pH increased to pH 8.3 with no lactone production. However, alcohol was produced in low yields (0.03g/l) showing that the dehydrogenase present is

reversible. It can be concluded that addition of the ketone to cells which have not reached pH 8 will not produce lactone due to the non expressed BVMO enzyme. However, addition of alcohol to cells with a low pH has a major effect on the expression of the BVMO enzyme where lactone production is observed, in a sense, the conversion of alcohol to ketone by this dehydrogenase initiates the fungal metabolism to express the BVMO enzyme during secondary metabolism.

During submerged culture of *C. echinulata* a decrease in DOT is observed followed by an increase in pH as discussed in chapter 2. It was noted at the end of the fermentation a alcoholic smell could be detected from the spent media. It would be plausible to assume that during the low DOT conditions the fungus was undergoing anaerobic fermentation resulting in the production of alcohol from the metabolism of pyruvic acid to ethanol which in turn induces the BVMO enzyme. However, the cause of the pH increase is still largely unknown, it can be assumed that this is caused by the conversion of chemical compounds either in the media or from the fungal metabolism itself.

Comparing the enzyme activity ratio between alcohol dehydrogenase and BVMO, between seven day and 24 hour shake flask growth gives the following ratios. The ratio in 24 hours growth is 2.5:1 (ADH:BVMO) whereas in seven day growth we see a ratio of 6:1 (ADH:BVMO). This larger ratio observed in the seven day growth may be attributed to the starting pH conditions and the length of culture time. As it is not practical to measure parameters such as DOT during shake flask culture it is difficult to understand the growth profile. However, it can be assumed that oxygen limitation is greater during shake flask culture and therefore fungus cultured for this length of time is, or has been subjected to anaerobic fermentation. This would explain the rapid production of lactone after 24 hours which was not observed in 24 hours shake flask culture that can be assumed has not undergone anaerobic growth conditions therefore, leading to a lower enzyme activity ratio.

5.4 Summary

In this chapter it has been shown that the alcohol dehydrogenase present in *C. echinulata* has an optimum working pH at 7 – 7.5, 1-1.5 pH units less than that of the optimum BVMO pH (*cf* pH 8-8.5) and is present in the fungus after 24 hours growth from shake flask culture.

Lactones have successfully been synthesised using bicyclo[3.2.0]hept-2-en-6-ol as a starting substrate using fungal mass grown for seven days reaching pH 7.44 that displays no BVMO activity at this pH. A time course experiment has shown the conversion of alcohol to ketone followed by lactone during which pH of the media increased. It has been hypothesised that during a 15L submerged culture the fungus undergoes anaerobic fermentation producing alcohol which in turn initiates the synthesis of the BVMO enzyme.

Substrate specificity of this alcohol dehydrogenase was not as successful as hoped. Out of the substrates used only cyclobutanol was converted in to cyclobutanone. However, the yield was somewhat low.
It is possible that a small ring size is necessary in order to fit in to the active site of the alcohol dehydrogenase and that any other ring systems larger than a four membered ring will not undergo conversion.

Attempts at the purification of the alcohol dehydrogenase were initiated but due to time limits complete isolation was not achieved. Isolation of this enzyme would provide a clean and convenient method of obtaining regioselective and stereospecific molecules.

CHAPTER 6

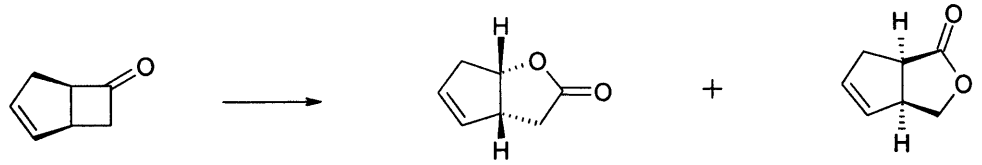
Regio- and enantio-selectivity in *C. echinulata*

6.1 Introduction

In the previous chapter the identification and characterisation of an alcohol dehydrogenase was discussed and its importance to the enzyme system present in *C. echinulata.*

In this chapter the regio and enantio selectivity of the alcohol dehydrogenase and the CeBVMO will be investigated. For the first time enantiomerically pure ketones, (+)-(1*R*,5*S*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one and (-)-(1*S*,5*R*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one will be used as substrates and the configuration of the lactone products will be investigated.

As discussed in chapter one the CHMO isolated from *A. calcoaceticus* has the ability to carry out Baeyer-Villiger oxidation and resolve the racemic bicyclic ketone (+/-) bicyclo[3.2.0]hept-2-en-6-one to yield two lactones, the expected "normal" lactone (-)-(1*S*,5*R*)-2-oxabicyclo[3.30]oct-6-en-3-one and the "abnormal" (-)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one respectively in a 1:1 ratio, figure 6.1



bicyclo[3.2.0]hept-2-en-3-one

2-oxabicyclo[3.3.0]oct-6-en-3-one
(-)-(1*S*,5*R*)

3-oxabicyclo[3.3.0]oct-6-en-2-one
(-)-(1*R*,5*S*)

Figure 6.1: Conversion of (+/-) bicyclo[3.2.0]hept-2-en-6-one to (-)-(1*S*,5*R*)-2-oxabicyclo[3.30]oct-6-en-3-one and (-)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one respectively in a 1:1 ratio

This system and reaction has been extensively studied and several theories exist to describe the stereoselectivity of the AcCHMO system which has become the model most widely used. The most common model is based on "cubic space" (figure 6.2) where an assumption has been made that the active site will be determined by the combination of stereochemical and stereoelectronic effects (Walsh *et al.*, 1988). Furstoss proposed a model based on this approach where "forbidden" areas exist where steric hindrance occurs within the cubic space (Alphand *et al.*, 1992). Three assumptions are used in this model; firstly, the flavin is occupied in a fixed position within the active site and therefore the terminal oxygen of the flavin hydroperoxide group must also be located at a fixed position. Secondly, the peroxyflavin should occupy an equatorial position and finally during the fragmentation of the tetrahedral intermediate the migrating carbon-carbon bonds all adopt the same orientation within the active site. Based on this model, only (-)-(1*S*,5*R*) and (-)-(1*R*,5*S*) lactone enantiomers can be formed.

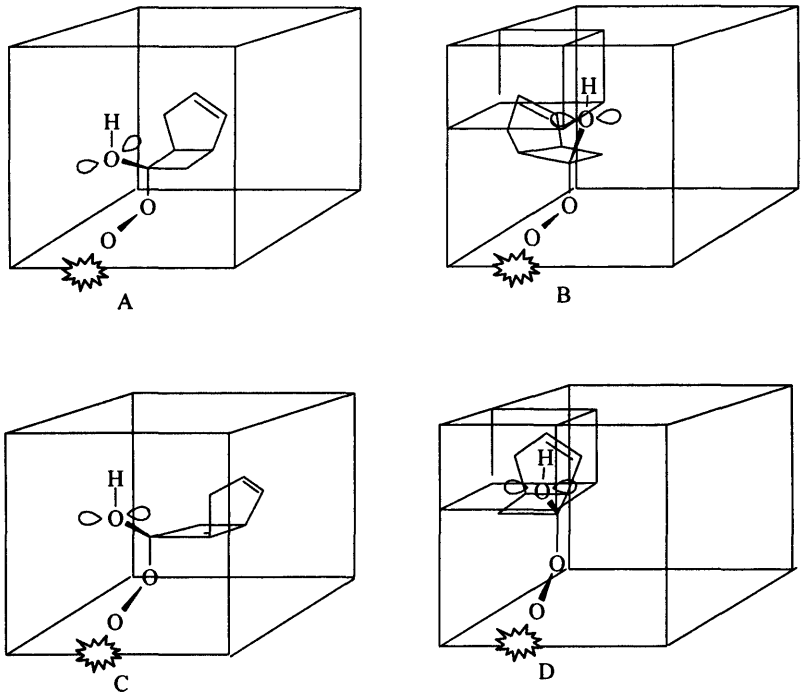


Figure 6.2: Active sight model for CHMO based on cubic space model proposed by Furstoss *et al.* (modified from Willetts 1997) ✸ :- flavin
A:- preferential formation of (1*S*,5*R*) lactone B:- Non permitted formation of (1*S*,5*R*) lactone
C:- preferential formation of (1*R*,5*S*) lactone D:- Non permitted formation of (1*R*,5*S*) lactone

However, the "cubic space" model is based on the AcCHMO system and it is known that other monooxygenases exists which display the mirror reaction. One such example is the 2,5-Diketocampahne monooxygenase (2,5-DKMO) isolated from *P. putida* that shows the opposite of this model only producing the (+)-(1*S*,5*R*) and (+)-(1*R*,5*S*) lactone enantiomers (Kelly *et al.*, 1998). The use of the AcCHMO and 2,5-DKMO enzymes provide access to all four lactones via enantiodivergent and enantiospecific resolution of the (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone.

However, *C. echinulata* provides access to the "abnormal" 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone via a regio and enantioselective oxidation.

To date, enantiomerically pure ketones have not been presented to the AcCHMO or CeBVMO. However, one could predict the outcomes that only (-)-(1*R*,5*S*) and (-)-(1*S*,5*R*) lactone enantiomers will be formed, figure 6.3.

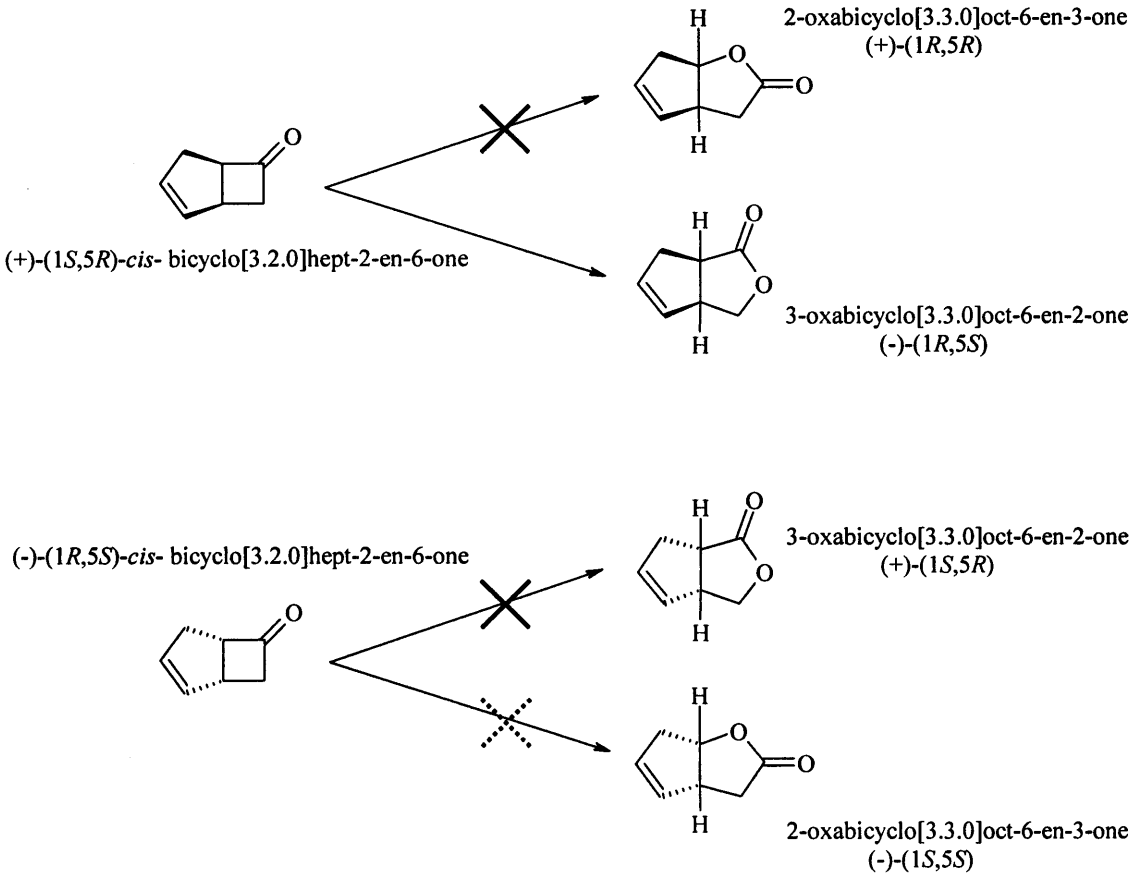


Figure 6.3: Predicted conversion of enantiomerically pure ketones in to corresponding lactones by CeBVMO (⁘) :- not favoured (✕):- no conversion

6.2 Materials and Methods

Unless otherwise stated all chemicals were of the highest purity available from Sigma-Aldrich Chemical Company (Poole, Dorset, UK) Growth media components were obtained from Oxoid Ltd (Basingstoke, Hants, U.K.) *Cunninghamella echinulata* NRRL 3655 was obtained from LGC (Teddington, Midds, UK).

6.2.1 Analytical Gas Chromatography (G.C.)

All metabolites were identified by comparison of their retention times using an XL-2 gas chromatograph with flame ionisation detector (Perkin-Elmer, Norwalk, CT, USA) fitted with a ZB1 non-polar dimethylsiloxane column (30 m × 0.25 mm × 0.25 μm) (Phenomenex, Macclesfield, Cheshire, U.K.) with helium as the mobile phase with those of authentic compounds synthesised by classical chemical reactions or commercially available. 400 μl supernatant from the biotransformation was extracted with an equal volume of ethyl acetate containing naphthalene as the internal standard. Standard GC operating temperatures were set as follows: column 110°C, injector 250°C and detector 250°C. Chiral analysis was performed by GC using a Chiraldex-B column (25 m × 0.25 mm × 0.25 μm) (SGE, Milton Keynes U.K.)with helium as the mobile phase with operating temperatures as follows: column 90°C 4mins followed by ramp at 15°C min to 120°C, injector 200°C and detector 200°C.

6.2.2 Fermentations

Fermentations were carried out using corn steep liquor media (CSL): CSL (20 g/L), glucose (4 g/L), KH_2PO_4 (1 g/L), K_2HPO_4 (2 g/L), $NaNO_3$ (2 g/L), KCl (0.5 g/L), $MgSO_4$ (0.5 g/L), $FeSO_4$ (0.02 g/L).

Fermentations were performed in 250ml non baffled shake flasks containing 100ml CSL which were inoculated using fungal spores taken from a CSL slope and incubated at 28°C, 200rpm over a period of 24 hours to seven days.

6.2.3 Bioconversions

In a typical procedure the fungal pellets were harvested by filtration through a Miracloth membrane (20-22 μm pore size) (Calbiochem, Notts, UK). 5 g of cells were re-suspended back into 50 ml of spent fermentation broth and incubated with 50 μl of substrate in a reciprocating shaker at 170 rpm, 28°C for 24 hours.

6.3 Results

6.3.1 Pure ketone bioconversion

Performing a bioconversion using separated enantiomers of bicyclo[3.2.0]hept-2-en-6-one ketone gave the following results.

In figure 6.4 it can be seen that using (+)-1R 5S-*cis*-bicyclo[3.2.0]hept-2-en-6-one leads to initial production of 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone after 24 hours. After a further 24 hours, 2-oxabicyclo[3.3.0]oct-6-en-3-one lactone is synthesised. Between 48 and 72 hours the rate of 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone production appears to level to a steady rate whereas 2-oxabicyclo[3.3.0]oct-6-en-3-one lactone shows a higher rate of production at 0.013 g/l/24h

In figure 6.5 it can be seen that using (-)-1S 5R-*cis*-bicyclo[3.2.0]hept-2-en-6-one ketone leads to the opposite as seen with (+) ketone. After 24 hours 2-oxabicyclo[3.3.0]oct-6-en-3-one lactone is observed. After a further 24 hours 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone is produced. Between 48 and 72 hours 2-oxabicyclo[3.3.0]oct-6-en-3-one lactone is not present, however, 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone shows a rate of production at 0.013 g/l/24h, an identical rate compared to that observed previously using (+) bicyclo[3.2.0]hept-2-en-6-one.

Previous experiments running a biotransformation using racemic (+/-) bicyclo[3.2.0]hept-2-en-6-one have shown that both lactones are produced after 24 hours with the enzyme induced 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone formed at a higher concentration compared to the 2-oxabicyclo[3.3.0]oct-6-en-3-one lactone (results not shown).

However, it is apparent from the results that greater quantities of lactone are produced when using (-)-(1*S*,5*R*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one ketone compared to that of the regio-plus enantiomer ketone, approximately three times more lactone produced, further strengthening published results that this BVMO is enantioselective.

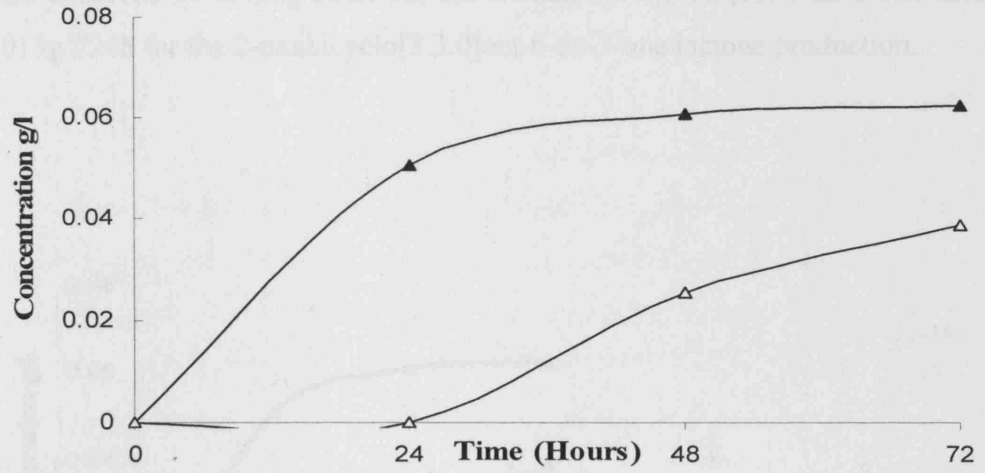


Figure 6.4: Bioconversion using (+)-(1*R*, 5*S*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one ketone using fungal mass grown for seven days performed in media ▲-3-oxabicyclo[3.3.0]oct-6-en-2-one lactone △ - 2-oxabicyclo[3.3.0]oct-6-en-3-one

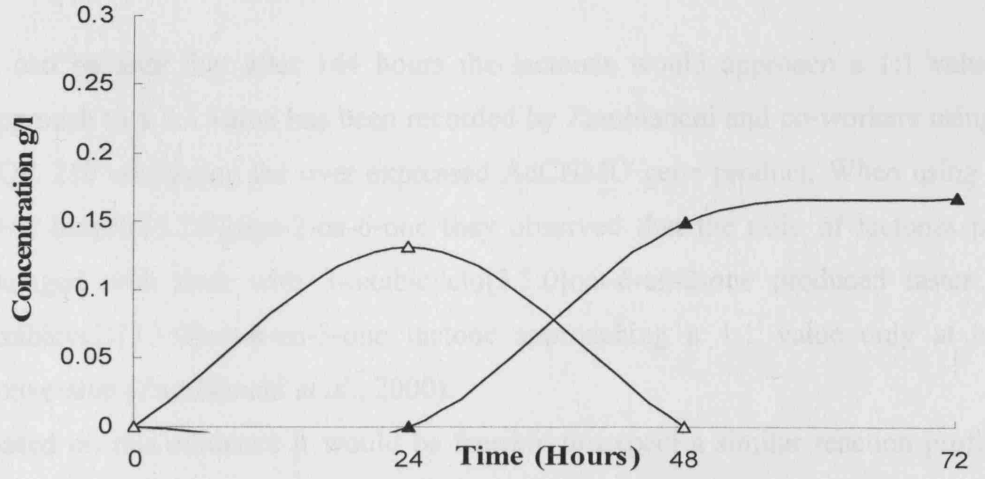


Figure 6.5: Bioconversion using (-)-(1*S*,5*R*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one ketone using fungal mass grown for seven days performed in media ▲-3-oxabicyclo[3.3.0]oct-6-en-2-one lactone △ - 2-oxabicyclo[3.3.0]oct-6-en-3-one

Below, figure 6.6 shows a predicted extrapolated plot of the lactone concentrations. This plot has been based on the assumptions from the previous experiments using the rates observed of 0.002g/l/24h for the 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone and 0.013g/l/24h for the 2-oxabicyclo[3.3.0]oct-6-en-3-one lactone production.

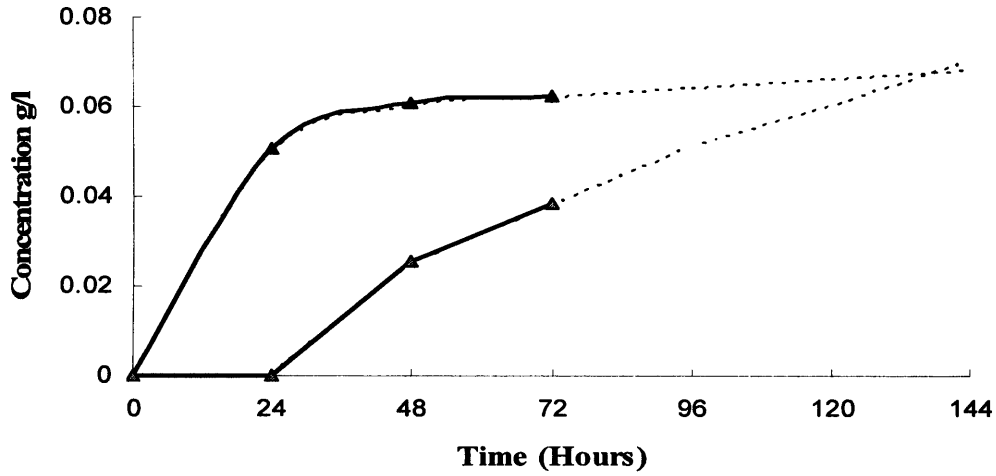


Figure 6.6: Theoretical extrapolation from 72 hours onwards from the bioconversion using (+)-(1*R*,5*S*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one ketone ▲-3-oxabicyclo[3.3.0]oct-6-en-2-one lactone (enzyme induced) △ - 2-oxabicyclo[3.3.0]oct-6-en-3-one (chemical) (.....) - extrapolated plot

It can be seen that after 144 hours the lactones would approach a 1:1 value. This approach to a 1:1 value has been recorded by Zambianchi and co-workers using *E. coli* pQR 210 containing the over expressed AcCHMO gene product. When using racemic (+/-) bicyclo[3.2.0]hept-2-en-6-one they observed that the ratio of lactones produced changed with time with 3-oxabicyclo[3.3.0]oct-6-en-2-one produced faster than 2-oxabicyclo[3.3.0]oct-6-en-3-one lactone approaching a 1:1 value only at complete conversion (Zambianchi *et al.*, 2000).

Based on this evidence it would be feasible to expect a similar reaction profile when using *C. echinulata* following the predicted plot of lactone concentration, figure 6.6.

However, the existence of two BVMO enzymes in *C. echinulata* is also a possibility with each producing highly regioselective lactone products.

As previously discussed in chapter 5 an alcohol dehydrogenase is present within the fungus converting the bicyclo[3.2.0]hept-2-en-6-ol alcohol in to bicyclo[3.2.0]hept-2-en-6-one ketone. However, the reverse reaction, ketone to alcohol has been observed. Below in figure 6.7 and 6.8 it can be clearly seen that using (+)-(1*R*,5*S*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one the *exo* form of the alcohol is synthesised whereas using (-)-(1*S*,5*R*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one the *endo* alcohol form is synthesised. This observation is very interesting as once again we can identify an enzyme which acts in a highly regioselective way. Classical chemical synthesis of the alcohol predominantly yields the *endo* alcohol form, i.e. the less sterically strained form. Here we see the exclusive synthesis of the *exo* alcohol form when using (+)-(1*R*,5*S*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one ketone, summarised in figure 6.9.

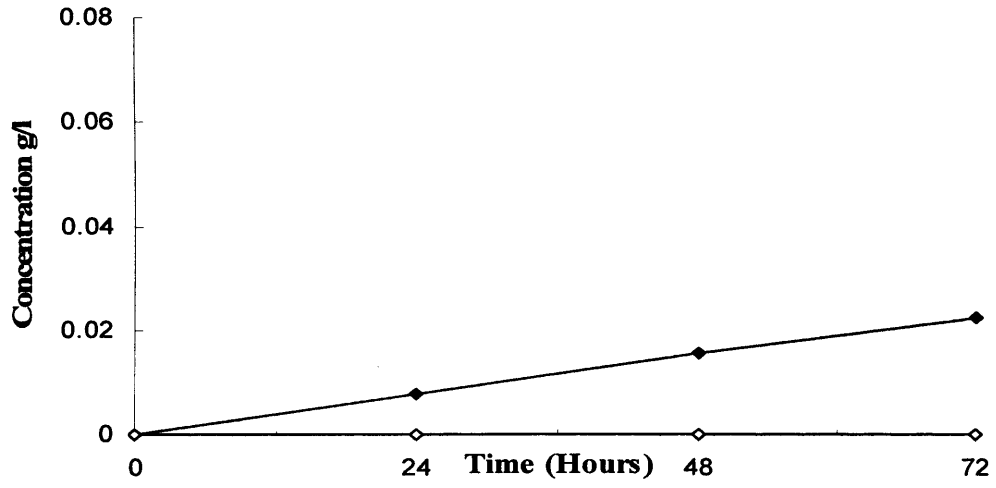


Figure 6.7: Bioconversion with (+)-(1*R*,5*S*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one ketone using fungal mass grown for seven days performed in media at pH 7.44. ♦- alcohol (exo) ◊- alcohol (endo)

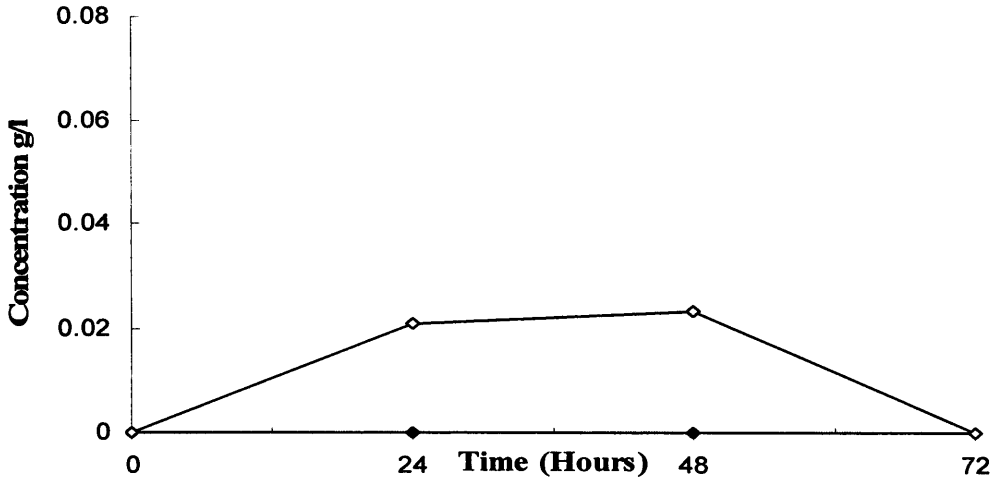
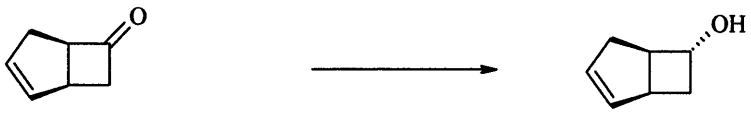
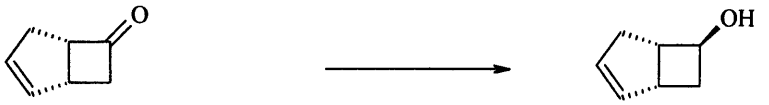


Figure 6.8: Bioconversion with (-)-(1*S*,5*R*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one ketone using fungal mass grown for seven days performed in media at pH 7.44. ♦- alcohol (exo) ◊- alcohol (endo)



(+)-1S 5R-*cis*- bicyclo[3.2.0]hept-2-en-6-one

exo bicyclo[3.2.0]hept-2-en-6-ol



(-)-1R 5S-*cis*- bicyclo[3.2.0]hept-2-en-6-one

endo bicyclo[3.2.0]hept-2-en-6-ol

Figure 6.9: Conversion of pure ketones in to corresponding bicyclo[3.2.0]hept-2-en-6-ols as observed from biotransformations.

Chiral analysis was performed on all bioconversion samples. However, the results for the production of lactones were inconclusive. It has been shown that (+)-(1*R*,5*S*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one gives rise to *exo* bicyclo[3.2.0]hept-2-en-6-ol and (-)-(1*S*,5*R*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one ketone gives rise to *endo* bicyclo[3.2.0]hept-2-en-6-ol respectively. However, it would be assumed that the enantiomers of each lactone would be produced from the corresponding ketone enantiomers. Initial investigation has revealed distinct lactone enantiomers being formed. As of yet these are unable to be confirmed until known standards are obtained.

6.4 Summary

From the results it has been shown that performing bioconversions using (+)-(1*R*,5*S*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one leads to initial production of 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone followed by 2-oxabicyclo[3.3.0]oct-6-en-3-one lactone after a further 24 hours. The opposite is seen using (-)-(1*S*,5*R*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one ketone where 2-oxabicyclo[3.3.0]oct-6-en-3-one lactone is observed first followed by 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone after a further 24 hours.
Both of the bioconversions show a rate of lactone production at 0.00054 g/l/h. However, three times more lactone is formed using the (-)-(1*R*,5*S*) ketone enantiomer compared to using the (+)-(1*S*,5*R*) ketone enantiomer.

Using the bicyclic ketones substrates for the alcohol dehydrogenase it has been observed that using (+)-(1*R*,5*S*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one leads to the formation of *exo* bicyclo[3.2.0]hept-2-en-6-ol whereas using (-)-(1*S*,5*R*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one *endo* bicyclo[3.2.0]hept-2-en-6-ol alcohol is formed.

CHAPTER 7

Discussion

7.1 Introduction

Ultimately, the main aim of this work was to examine the usefulness of the Baeyer-Villiger monooxygenase from *C. echinulata* and to map a path forward for scalable exploitation. This would involve the isolation and purification of the CeBVMO protein leading to crystallisation and structure elucidation. In order to reach this target the growth characteristics of this fungus were addressed enabling effective growth on a large scale to obtain maximum enzyme yields for extraction and purification.

However, throughout this work the project has identified several enzymes systems, each of which was investigated to uncover their relationship with the CeBVMO system. Consequently, this project has enabled many routes to be investigated which have led to many interesting results. However, the potential for further examination is highly desirable as an ultimate goal would finally isolate the CeBVMO and use it as a complementary system alongside theAcCHMO system.

7.2 *C. echinulata* for multistep biocatalysis

To date, most enzymes systems studied have used a single enzyme to perform specific bioconversions. These enzymes have either been used in the wild type organism or more commonly cloned and over expressed in an alternate host organism such as the CHMO from *A. calcoaceticus*. As previously discussed, the AcCHMO was removed from the enzyme pathway and exploited for its ability to perform oxygenation reactions.

Due to the pathogenicity of *A. calcoaceticus* (class II) this wild type enzyme system would not warrant commercial scale up. In addition, the wild type reaction breaks down the lactone products from the AcCHMO reaction.

In this thesis it has been shown that *C. echinulata* follows the same pathway as *A. calcoaceticus* showing the ability to perform three conversions on separate substrates using three different enzymes; an alcohol dehydrogenase, Baeyer-Villiger monooxygenase and a lactone hydrolase, figure 7.1.

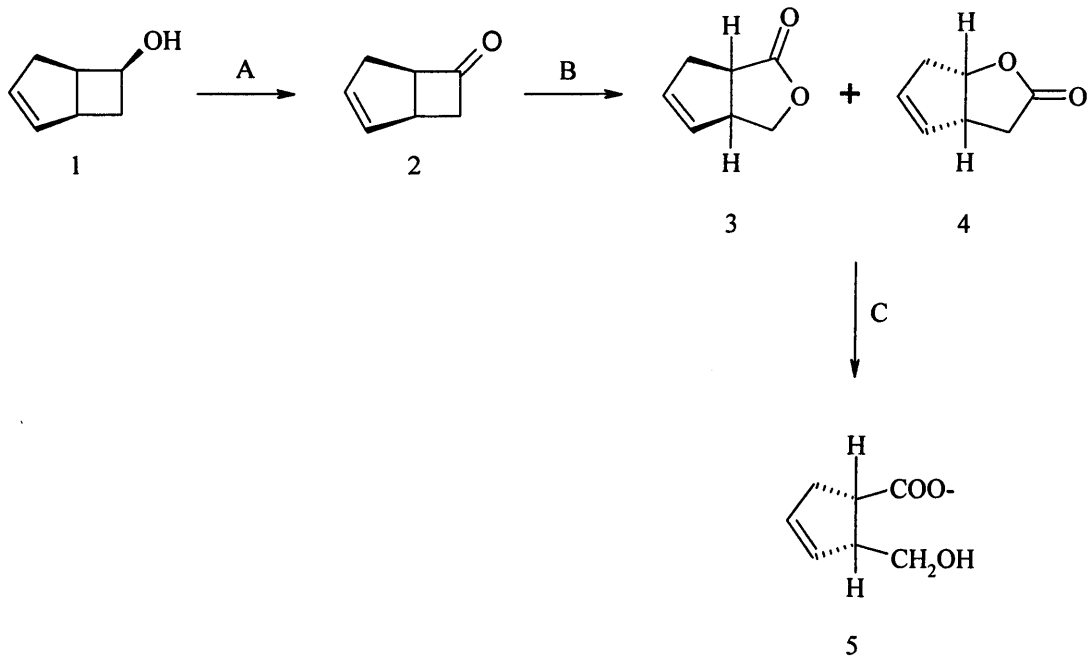


Figure 7.1: Degradation pathway of bicyclo[3.2.0]hept-2-en-6-ol.
1:- bicyclo[3.2.0]hept-2-en-6-ol 2:- (+/-) bicyclo[3.2.0]hept-2-en-6-one 3:- 3-oxabicyclo[3.3.0]oct-6-en-2-one 4:- 2-oxabicyclo[3.3.0]oct-6-en-3-one 5:- 2-hydroxymethyl-cyclopent-3-enecarboxylic acid A:- alcohol dehydrogenase B:- BVMO C:- lactone hydrolase

The BVMO present in *C. echinulata* provides a powerful tool in the synthesis of regio and enantio pure molecules. The conversion of racemic (+/-) bicyclo[3.2.0]hept-2-en-6-one ketone by *C. echinulata* is well documented. However, the bioconversion of enantiomerically pure ketones was described for the first time in this thesis. However, experiments to elucidate the resulting lactone enantiomers proved inconclusive.
The use of these enantiospecific ketones provides a valuable starting point in the synthesis of pure lactone products, with emphasis on the production of 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone which by conventional chemical methods proves challenging.

Historically, *C. echinulata* has always been cultured for a period of eight or more days in order to obtain BVMO activity. However, one important discovery during this project is the ability to grow *C. echinulata* for a 24 hour period and follow the reaction pathway starting with alcohol as an initial substrate. This reduced growth time holds huge potential as it eliminates time consuming fermentations.
One can therefore take a further step back in the reaction pathway to the alcohol dehydrogenase. It is known that the alcohol dehydrogenase present can work in reverse converting ketone in to alcohol and it has been shown that using (+)-(1*R*,5*S*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one leads to the formation of *exo* bicyclo[3.2.0]hept-2-en-6-ol whereas using (-)-(1*S*,5*R*)-*cis*-bicyclo[3.2.0]hept-2-en-6-one *endo* bicyclo[3.2.0]hept-2-en-6-ol alcohol confirming that the alcohol dehydrogenase enzyme acts in a highly regioselective way. It would therefore be feasible to start with one alcohol epimer and direct the reaction to produce enantio pure ketone and lactone products, figure 7.2. Directing the reaction in this way would be highly desirable and of commercial interest as the outcome of the reaction could be directed and controlled.

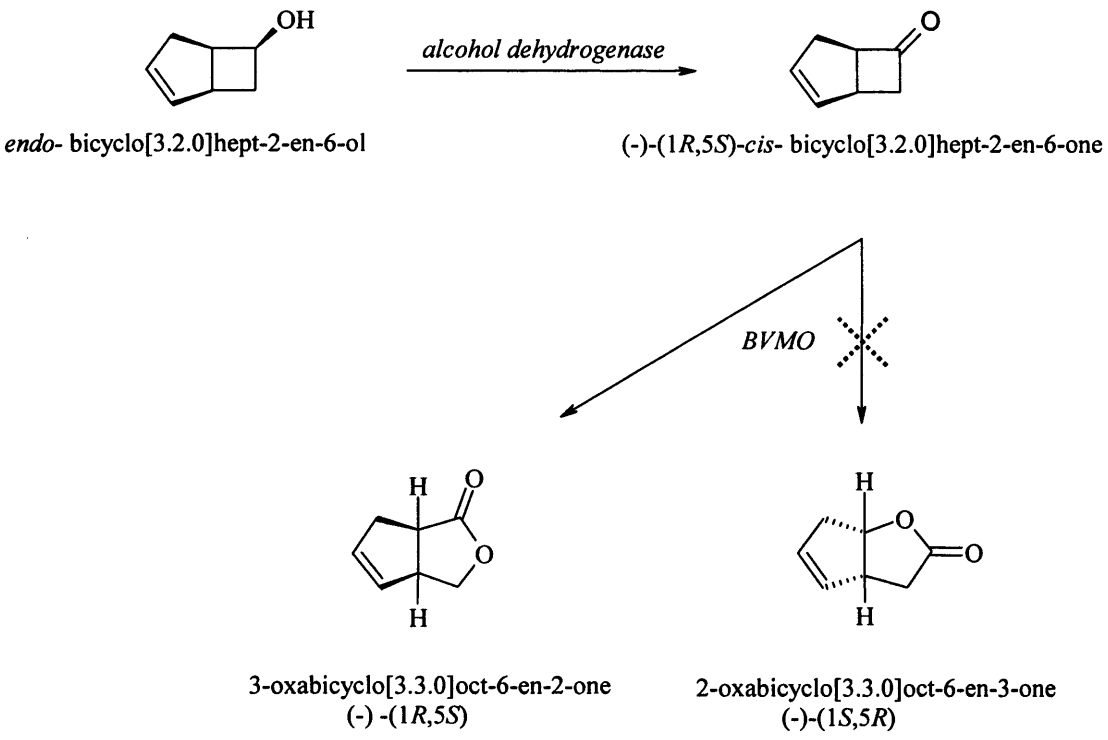


Figure 7.2: Controlling the reaction using a specific starting substrate alcohol to direct the reaction yielding corresponding enantiospecific lactone stereoisomers (⁘):- not favoured

This multi step reaction could be taken one step further by utilising the lactone hydrolase enzyme. In chapter four the presence of a lactone hydrolase from *C. echinulata* was discussed and its effect on the lactone regioisomers. Initial results showed that both lactones were acted upon by the lactone hydrolase. However, work carried out on the hydrolase inhibition showed that this hydrolase enzyme shows a high specificity towards the enzyme induced 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone.

With respect to this specificity this hydrolase could be used as a convenient separation method of the two lactones. The resulting ring cleaved lactone could be ring closed using di phenyl amine followed by H^+ ion exchange chromatography. However, as this lactone hydrolase purely targets the 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone, which is predominantly produced during the reaction it would be more desirable to inhibit this enzyme and separate the residual lactone regioisomer using classical methods in order to limit the loss of the lactone yield and the chirality of the molecule.

The above multistep reaction described utilises the three pathway enzymes within the whole cell environment. Using *C. echinulata* as a whole cell biocatalysts removes the requirement for cofactor supply that would be necessary if this was carried out as a single pot reaction using isolated enzymes.

Using this whole cell enzyme pathway system it may be possible to perform batch fermentation and carry out the bioconversion reaction of a continuous feed system in combination with a continuous extraction system. It was found that fungal mass at pH 8-8.5 and displaying CeBVMO activity could be held in the fermenter for a further five days without displaying and significant loss in activity. However, after five or more days the pellets begin to lyse and activity is lost. It has been shown that the CeBVMO can be induced by the addition of alcohol and it would therefore be possible to utilise the fungus after 1-2 days growth during submerged culture therefore extending the window of opportunity for bioconversion. It has been shown by Hilker and co-workers (Hilker *et al*, 2004) that the use of an adsorbent resin (Optipure L-493) it is possible to feed the substrate and remove the product simultaneously using a process known as "substrate feeding and product removal" (SFPR). Using this principle it may be possible to screen for resins that selectively bind ketone, alcohol or lactone only therefore providing a convenient method of product removal at both stages of the multi step pathway thus providing access to enantio pure ketone and regio and enantio pure lactone. An example of a continuous feed system is shown in figure 7.3. Using *C. echinulata* in this system it would be possible to start with either alcohol or ketone as starting substrates and selectively remove lactone along with ketone and/ or alcohol respectively.

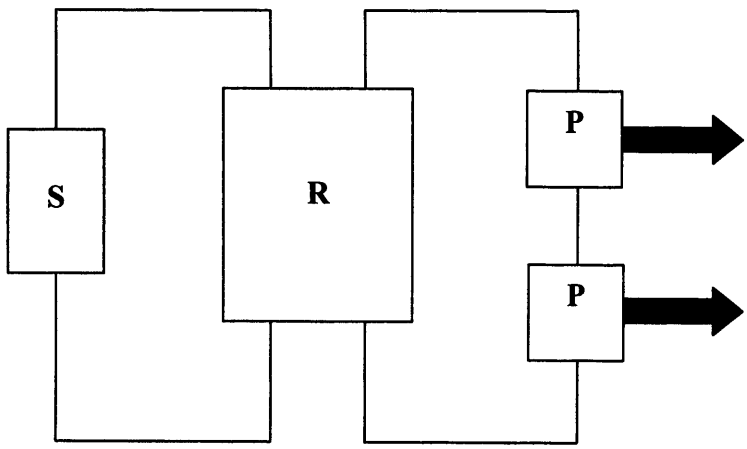


Figure 7.3: Example of continuous feed system. R= reactor S= substrate P= product

However, scaling up this multistep reaction is one possibility but the use of *C. echinulata* as a small scale screening system is also viable. The use of the ADH, which is known to be active after 24 hours growth and highly selective, would provide a useful tool in the conversion of small ring alcohols to ketones which may potentially provide new regio and enantiospecific ketone precursors.

Throughout this thesis there has been an emphasis on the need to clone this CeBVMO in to an alternate host organism. However, since the discovery of the ADH enzyme pathway this has highlighted the use of *C. echinulata* as a whole cell biocatalyst exploiting its ability to perform a multistep bioconversion reaction. To clone and over express this CeBVMO on its own would provide a complementary system to the existing cloned AcCHMO providing access to single regio lactone isomers. Currently one disadvantage of the CeBVMO from *C. echinulata* is the low lactone yield, producing 0.5 g/L of lactone over a 24 hour period, due to low expression of the CeBVMO enzyme. A major advantage of cloning this enzyme would be to increase the speed of reaction and lactone yield.

However, the use of the ADH also provides an extremely powerful biocatalytic tool. It would therefore be advantageous to clone this ADH along with the BVMO in to an alternate host organism, such as *E. coli*, thus providing the stability of a using whole cell system complete with cofactor recycling along with the option to start with two substrates, alcohol or ketone. This in turn would allow a large scale SFPR system a reality as the engineering of such reactions is well known along with the growth characteristics of *E. coli*.

CHAPTER 8

8 Conclusions

- Understanding of fungal growth characteristics during solid and submerged culture. Successful growth on a 15L scale with the following conditions; Airflow 0.46 vvm (7 L/min), speed 450rpm, baffles removed using an inoculum concentration of $2x10^8$ fugal spores yielding 0.43 g/L 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone and 0.02 g/L 2-oxabicyclo[3.3.0]oct-6-en-3-one lactone.

- *C. echinulata* has a narrow substrate specificity where small ring cyclobutanone and cyclobutanol were favoured by the CeBVMO and ADH respectively.

- The BVMO from *C. echinulata* is shown to be NADPH dependent therefore a Type 1 monooxygenase and has an optimum working pH range between 7 and 8.

- Partial purification of the CeBVMO has shown that the CeBVMO lies between 50 and 70KDa respectively, similar to the isolated AcCHMO which has a molecular weight of 60KDa.

- A lactone hydrolase has been isolated and has been shown to be specific for the 3-oxabicyclo[3.3.0]oct-6-en-2-one lactone which can be inhibited using Alloxan or E-64, both of which target cysteine residues.

- Alcohol dehydrogenase from *C. echinulata* identified and shown to have optimum working pH range between 7 and 8. Shown to be present in the fungus after 24 hours growth by shake flask culture.

- The CeBVMO can be induced using bicyclo[3.2.0]hept-2-en-6-ol alcohol where a reduced growth time from eight days to 24 hours is necessary to promote the pathway enzymes.

- It has been shown that using enantiopure bicyclo[3.2.0]hept-2-en-6-one ketone the following conversions are observed.
  (+) bicyclo[3.2.0]hept-2-en-6-one yields *exo* bicyclo[3.2.0]hept-2-en-6-ol
  (-) bicyclo[3.2.0]hept-2-en-6-one yields *endo* bicyclo[3.2.0]hept-2-en-6-ol

CHAPTER 9

9 Future work

9.1 Growth and substrate specificity of the BVMO from *C. echinulata*

In order to investigate whether pH or dissolved oxygen tension directly affects the CeBVMO induction two separate experiments could have been run. The first, running the fermentation at constant DOT and the second, running at constant pH. As these two parameters are fundamental in the culture of the fungus controlling these would give an indication as to the factor affecting the 'switching on' of the CeBVMO enzyme. Maintaining a constant DOT would show if the pH change is caused due to anaerobic fermentation or if the production of secondary metabolites leads to this pH increase. Maintaining a constant pH would show if this was an influencing factor in CeBVMO induction or whether it was due to an anaerobic fermentation period.

Investigating the natural function of the CeBVMO enzyme would allow a further understanding of this useful enzyme. To date, the natural function remains unknown. One set of experiments looking at possible secondary metabolites produced during fermentations and applying those to the CeBVMO enzyme could have allowed some insight to its vital role within *C. echinulata* and other Baeyer-Villiger type producing organisms.

Continuous culture of the fungus on a 15L scale, allowing investigation in to the life and availability of the enzyme pathway. Investigate continuous extraction from the reaction removing ketone and lactone products during a bioconversion reaction in-situ.

9.2 Protein purification of the BVMO

Isolate and purify CeBVMO protein obtaining N terminus sequence data which would allow subsequent design of oligonucleotide primers to isolate BVMO DNA sequence. Ultimately, to clone and over-express the CeBVMO enzyme in an alternate host allowing further investigation and understanding of the CeBVMO enzyme applications.

9.3 Isolation and purification of the lactone hydrolase enzyme

Isolate and purify lactone hydrolase. Add next purification step using thiol sepharose 4B as this will bind to the lactone hydrolase enzyme as it appeared to utilise cysteine residues as part of the catalytic active site.

Assay development combining *para*-nitrophenyl acetate reaction with a specific assay monitoring the breakdown of the lactones.

9.4 Identification of an alcohol dehydrogenase

Confirming the presence of ethanol during submerged culture leading to investigation of inducing the BVMO with the addition of ethanol during submerged culture.

Investigate a wider range of substituted cyclobutanols to determine if the size of the substitution has an effect on the enzymes ability to perform a bioconversion.

9.5 Regio and enantioselectivity in *C. echinulata*

Repeating the bioconversion experiments using enantiopure ketones to detect the absolute configuration of the lactone produced.

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APPENDIX I

Isolation of the BVMO using degenerate primers

Throughout this project many approaches to isolating the Baeyer-Villiger monooxygenase have been studied. Some approaches have yielded successful results that have been discussed. However, others have not, either due to technical problems encountered along the way or time constraints on the project.

i) Introduction

BVMOs have been isolated in many different organisms. However, only a handful have been isolated and cloned providing valuable sequence data (table A.1).

Table A.1: BVMO that have been cloned and sequenced.

Organism	Reference
Cyclohexanone monooxygenase from *Brevibacterium*	Brzostowicz *et al*, 2000
Cyclohexanone monooxygenase from *A. calcoaceticus*	Chen *et al*, 1988
Cycohexanone-1,2-monooxygenase from *Exophiala jeanselmei*	Hasegawa *et al*, 2000
Hydroxyacetophenone monooxygenase from *Pseudomonas fluorescens*	Kamerbeek *et al*, 2001
Cyclodecane monooxygenase from *Rhodococcus rubber*	Kostichka *et al*, 2001

Cloning BVMO genes is seen as a worthwhile exercise as most BVMOs are part of a degenerate system in the natural host, for example, in the wild type *A. calcoaceticus* a lactone hydrolase is present which degrades the natural products produced. Therefore it is advantageous to remove unwanted enzymes.

It has been found that some organisms contain several BVMOs which leads to reduced selectivity if they share the same substrate specificity (Van Beilen and co workers, 2003).

The organisms *Acinetobacter* sp. NCIMB 9871 BVMO, *Rhodococcus Rhodochrous* IFO3338, steroid monooxygenase and putative BVMO sequence from *Pseudomonas fluorescens* DSM 50106 were used to design highly degenerate primer sequences (Van Beilen *et al*, 2003). The forward sequence was based on a conserved region in the FAD binding Rossman fold. The reverse sequence was based on a NADPH binding Rossman fold. Using this method several novel BVMO sequences have been identified from *Comamonas* sp. NCIMB 9872, *R. Rhodochrous* DSM 11097 and *Xanthobacter* sp. ZL5. From this study it has been shown that all BVMOs contain a FAD binding signature (G(A,G,S,TGX(A,G,S,T)G) and an adenosine binding signature (GXGXXG).

ii) Genomic DNA extraction

100ml of corn steep liquor broth (in a 250ml shake flask) was inoculated with spores taken from a slope and grown for 3 days until pellets had formed. 5g of fungal mass was removed and the mycelium disrupted using two different methods: A) Fungal mass ground in a cooled mortar containing liquid nitrogen and a sufficient amount of acid washed sand and glass beads (100 mesh BDH 15032) and ground for approximately three minutes until a biscuit like texture was achieved. B) Fungal mass suspended in 20mls 100mM phosphate buffer (pH 8) and sonicated on ice at 18μ, 15 cycles of 30s with 15s rest period.

iii) Sigma and Qiagen plant genomic DNA extraction kits

Sonicated fungal mass was centrifuged (4000rpm, 10min, rt). The supernatant was removed and the top layer (approx 5mm) of the pellet containing the fungal protoplasts was removed and the genomic DNA was extracted using the commercially available kits, which provides a fast extraction method for maximum DNA removal and recovery. SDS based lysis solutions release the DNA and using filtration and binding columns DNA can be obtained in high quality and yields. The approximate purity and concentration of the DNA sample was determined by measuring the optical density at A_{260} and A_{280}. The approximate size of the DNA was measured by running the sample on a 1% agarose gel.

iv) DNA extraction using hexadecyl trimethyl ammonium bromide (CTAB)

Fungal mass (200mg) which had either been ground and sonicated was added to 1ml CTAB buffer and thoroughly vortexed. After incubation at 65°C for 2h, 500µl Phenol:Chloroform:Isoamyl alcohol (24:24:1, PCI) was added and vortexed briefly. After centrifugation (13,000rpm, 15min, rt). 300µl of the top aqueous phase was removed and transferred to a new tube. DNA was precipitated by the addition of 10µl 3M sodium acetate (pH 5.2) and 0.5 volumes of cold (-20°C) ethanol (100%, v/v) and left at –20°C overnight, after which it was recovered by centrifugation (13,000rpm, 30min, rt). The pellet was rinsed once with 70% (v/v) ethanol and the tube incubated at room temperature until dry. The pellet was resuspended in 115µl deionised water and incubated with 4µl RNAse A at 37°C for 15min and finally quantified.

CTAB Buffer:-
- 2% (w/v) CTAB
- 100mM Tris HCl
- 10mM EDTA
- 0.7M NaCl
- 2% (w/v) SDS
- 1% (v/v) 2-mercaptoethanol
- Solution pH at 7.5

v) DNA extraction using alkaline lysis

Fungal mass (250mg) which had been sonicated was added to 200µl alkaline lysis buffer at 90°C for 15min, after which 400µl lysis buffer was added and heated at 60°C. After 1h the DNA was extracted using PCI (as detailed above)

Alkaline lysis buffer:- NaOH , 50mM in deionised water

Lysis buffer:-
- 50mM Tris HCL
- 50mM EDTA
- 3% (w/v) SDS
- 1% (v/v) 2-mercaptoethanol

vi) DNA extraction using lysing enzymes

A variety of lysing enzymes were used to degrade the fungal protoplasts. The table below shows which enzymes were used and amounts added to 250mg of ground fungal mass. The buffer used throughout was 100mM sodium phosphate buffer containing 1.2M $MgSO_4$, pH 5.8. 400µl of buffer was added to each reaction and left overnight at 37°C with gently shaking.

Table A.1: Lysing enzymes used and sources.

Lysing Enzyme	Final concentration in reaction
Lysing enzymes (from *Trichoderm harzianum*)	0.025mg
Lyticase (from *Arthrobacter luteus*)	20mg
Protease E (from *Streptomyces griseus*)	10mg
Protenase K (from *Tritirachium album*)	24 units

vii) DNA extraction using modified alkaline lysis method

Fungal mass (13g, wcw) was suspended in 30mls alkaline lysis buffer (described previously section XXX) and sonicated on ice for 15 cycles at 18µ, 30sec with 10sec rest period. After incubation at 60°C for 2h, 1ml PCI was added and gently mixed for 10min. After centrifugation (13,000rpm, 15min, rt). 500µl of the top aqueous phase was removed and transferred to a new tube. 500µl Tris EDTA (TE) buffer (10mM Tris HCl (pH 8), 1mM EDTA) was added back to the organic phase to re-extract the remaining white precipitate that had formed. Both the aqueous phases were pooled and a further PCI extraction was performed. 20µl sodium acetate buffer (3M) was added to the aqueous phase along with 1ml chloroform, mixed gently and centrifuged (13,000 rpm, 5min, rt). DNA was precipitated by the addition of 20µl 3M sodium acetate (pH 5.2) and 2.5 volumes of cold (-20°C) ethanol (100%, v/v) and left at –20°C for 30min, after which it was recovered by centrifugation (13,000rpm, 30min, rt). The pellet was rinsed once with 70% (v/v) ethanol (rt) and the tube incubated at room temperature until dry. The pellet was resuspended in 80µl deionised water and incubated with 4µl RNAseA at 37°C for 15min and finally quantified.

viii) Agarose gel electrophoresis

Routine DNA identification was carried out using a 1% agarose gel. The gels allowed the identification of the desired DNA fragments and the existence of other contaminating DNA. The apparatus used for this technique was obtained from Amersam Phamacia Biotech, Bucks, UK.

Buffers used:
Tris Acetate EDTA (TAE) buffer (10× conentrate): Tris Base ($48.2gL^{-1}$), EDTA ($3.72gL^{-1}$), glacial acetic acid (11.42ml) made up to 1 litre with water.
Sample buffer: Bromophenol blue (0.25% w/v), Sucrose (40% w/v), Xylene Cyanole (0.25% w/v)

Agarose (1g) was added to TAE buffer (100mls, 1× concentrate) and microwaved until boiling to melt agarose. The molten agarose was allowed to cool to approx. 60°C. Ethidium bromide was added to a final concentration of 0.5µg/ml. The gel was poured in to a casting tray containing a 10 well comb was added and allowed to set.
Samples (10µl) were mixed with sample buffer (4µl) and loaded on to the gel along with suitable DNA markers for size comparison. The samples were run at 60V (constant) for 45mins. The DNA was visualised under UV light.

ix) Polymerase chain reaction (PCR)

The reaction mixture for a standard PCR consisted of:

1-5µl	Template DNA (100ng genomic)
5µl	PCR buffer (10× concentration)
5µl	10mM dNTP mix
5µl	Primer 1 (5'-GGIACITGGCCATGGAAYIIITAYCCIGG-3')
5µl	Primer 2 (3'-TTYKCACAICTGCAGTAICCITG-5')
0.5µl	2.5 unit μ^{-1} Taq poloymerase
DNAse-free water to 50µl	

PCR reactions were carried out in a Techne PHC-3 thermal cycler. The program consisted of a number of steps detailed in table A.2.

Table A.2: PCR running conditions.

Step	Temperature	Time	Number of cycles
Initial denaturation	94°C	4 min	Once
Denaturation	94°C	45 sec	
Annealing	48°C	1 min	25 cycles
Elongation	72°C	30 sec	
Final extension	72°C	4 min	Once

x) PCR optimisation

The reaction mixture for a standard PCR consisted of:

10µl	Template DNA (100ng genomic)
1µl	Universal buffer (50× concentration)
5µl	PCR buffer (10× concentration) from Sigma kit
1µl	10mM dNTP mix
5µl	Primer 1 (5'-GGIACITGGCCATGGAAYIIITAYCCIGG-3')
5µl	Primer 2 (3'-TTYKCACAICTGCAGTAICCITG-5')
0.5µl	2.5 unit μ^{-1} Taq poloymerase

DNAse-free water to 50µl

Table A.3: PCR optimisation conditions.

Step	Temperature	Time	Number of cycles
Initial denaturation	94°C	4 min	Once
Denaturation	94°C	45 sec	
Annealing	48°C	0 min	25 cycles
Elongation	48 °C to 72°C (0.5°C s^{-1})	4 mins at 72°C	
Final extension	72°C	4 min	Once

Table A.4: Sigma PCR optimisation kit buffers.

Buffer number	Buffer composition (10× concentration)
1	100mM Tris-HCL (pH 8.3), 15mM $MgCl_2$, 250mM KCL
2	100mM Tris-HCL (pH 8.3), 15mM $MgCl_2$, 750mM KCL
3	100mM Tris-HCL (pH 8.3), 35mM $MgCl_2$, 250mM KCL
4	100mM Tris-HCL (pH 8.3), 35mM $MgCl_2$, 750mM KCL
5	100mM Tris-HCL (pH 8.8), 15mM $MgCl_2$, 250mM KCL
6	100mM Tris-HCL (pH 8.8), 15mM $MgCl_2$, 750mM KCL
7	100mM Tris-HCL (pH 8.8), 35mM $MgCl_2$, 250mM KCL
8	100mM Tris-HCL (pH 8.8), 35mM $MgCl_2$, 750mM KCL
9	100mM Tris-HCL (pH 9.2), 15mM $MgCl_2$, 250mM KCL
10	100mM Tris-HCL (pH 9.2), 15mM $MgCl_2$, 750mM KCL
11	100mM Tris-HCL (pH 9.2), 35mM $MgCl_2$, 250mM KCL
12	100mM Tris-HCL (pH 9.2), 35mM $MgCl_2$, 750mM KCL
	Universal buffer (50× conc) 20mM Tris-HCL (pH 8.0), 250nm EDTA

xii) Results

Extraction of DNA from the fungus has been a time consuming process. Many attempts were made using different strategies ranging from commercial kits to phenol-chloroform extraction. One of the biggest problems was efficiently breaking open the fungal pellets due to the biochemical structure of the fungal cell wall. Lysing enzymes were used but proved unsuccessful. However, sonication was deemed to be the most efficient method. Once the fungal wall was broken open the protoplasts containing the DNA were collected by centrifugation and were harvested from the top layer of the pellet. Commercial DNA extraction kits gave the most reliable and consistent results yielding clean high molecular weight DNA unlike DNA obtained using the standard phenol-chloroform extractions.

xii) PCR reactions

Initial PCR reactions were carried out according to Van Beilen *et al*, 2003 and no PCR products were visible after the cycle. Further cycles were run using DNA from the first reaction as a template. However, this to proved unsuccessful. This result was not surprising as PCR reaction conditions often vary greatly. Attention turned to finding a PCR reaction condition that worked, this would start by identifying conditions that gave non-specific binding, i.e. small non-coding fragments of DNA. Consideration was also given to the source of DNA polymerase that would be used. Standard Taq-DNA polymerase was initially used. However, as the DNA was large genomic DNA Sigma's RedTaq genomic DNA polymerase and Deep Vent DNA polymerase (New England Biolabs) also used.

APPENDIX II

Shotgun cloning of the lactone hydrolayse

i) Genomic DNA extraction

Spores were grown for 8 days until a pH 8 was attained and the culture was then harvested. The fungal mass was removed and ground in liquid nitrogen. Genomic DNA was extracted using Sigma G2N10 plant genomic DNA extraction kit which provides a fast extraction method for maximum DNA removal and recovery. Lysis solutions release the DNA and using filtration and binding columns DNA can be obtained in high quality and yields. The approximate purity and concentration of the DNA sample was determined by measuring the optical density at A_{260} and A_{280}.

ii) Vector digest

pGEM-3Z vector digested with *Bam*H1 overnight at 37°C. To prevent recircularisation of the cut vector, phosphate groups at each 5'-termini were removed using phosphatase alkaline, shrimp (Roche Diagnostics)

To clean up the DNA from the enzymatic reaction the digested vector fragments were run through a Qiagen MiniElute reaction clean up kit (Qiagen Ltd, Crawley, Sussex, UK).

iii) Ligation of Genomic DNA in to pUC18 expression vector

Genomic DNA fragments from the *Sau*3A digests were inserted in to the digested pGEM-3Z expression vector.

A series of ligation reactions were set up using 1µl of vector with insert DNA volumes varying from 3µl to 0.0001µl. Following transformation in to competent *E. coli JM109* cells, the optimum insert volume was chosen to produce approximately 2000 transformant colonies on a single TSB agar plate. Ligation was then repeated under these optimised conditions to produce an entire genomic library on 40 agar plates.

iv) Transformation of competent cells

A 200µl aliquot of competent cells was transferred in to a cooled microfuge tube. A 1µl plasmid DNA sample was added, mixed gently and incubated on ice for 30min. The reaction mixture was then heat shocked at 42°C for 1min followed by 4°C for a further 2min. 450µl of TSB broth was added, mixed and incubated at 37°C, 150rpm, 45min. The sample was then centrifuged at 700g, 1min to pellet the cells. 500µl supernatant removed, followed by resuspension of the deposit in the remaining supernatant. The entire mixture was spread on a to a TSB agar plate containing ampicillin (100µg/ml), IPTG (240µg/ml) and X-gal (1.6mg/ml) and incubated at 37°C overnight.

v) Blue-White selection of transformed cells and creation of master library plates

After overnight incubation colonies which appeared white on the agar plates were picked and placed in to individual wells on a 96 well plate containing TSB and ampicillin (100µl total volume) and incubated at 37°C, 1200rpm for a further 24 hours after which glycerol (50%, 100µl) was added to each well and frozen at -80°C and used as master stock culture library.

vi) Library screen for lactone hydrolayse and BVMO enzymes

20µl of cells taken from each well of the master stock culture and transferred to a fresh plate containing 50µl TSP/ ampicillin mix and incubated at 37°C, 1200rp for 24 hours. The contents of the wells were split and transferred in to two new sets of plates and reactions set up to lactone hydrolayse and BVMO as follows.

vii) Screening for lactone hydrolayse activity

50µl of cells from each well were added to 50µl sodium acetate buffer (25mM, pH 6). 50µl of pNPHOAc (10mM stock solution in ethanol) was added and the plate sealed and incubated at 37°C, 1200rpm for 10 minutes.

Esterase activity was monitored by the colour change from colourless to bright yellow. Wells which had turned yellow were traced back to their corresponding master stock plate wells and sub-cultured a further time (as above). 50µl of cells were taken and 0.5µl 3-oxabicyclo[3.3.0]oct-6-en-2-one (1mg/µl stock solution) was added and incubated at 37°C, 1200 rpm. After 24 hours ethyl acetate (130µl) was added to each well and extracted. Analysis was performed by GC.

APPENDIX III

List of suppliers

Acquisition Systems
Unit 4
Ancells Court
Anfirth Court
Fleet
GU13 8UX

Aldrich Chemical Company
The Old Brickyard
New Road
Gillingham
Dorset
SP8 4JL

Amersham Biosciences UK Ltd
Pollards Wood
Nightingales Lane
Chalfont St. Giles
Bucks
HP8 4SP

Bio-Rad Laboratories Ltd
Maylands Avenue
Hemel Hempstead
Hartfordshire
HP2 7TD

Bioprocess Engineering Services
186 Carver Drive
Sittingbourne
Kent
ME9 8NP

Calbiochem
Padge Road
Beeston
Nottingham
NG29 2JR

Ingold Mettler Toledo AG
Im Langacher
8606 Greifensee
Switzerland

LGC
Queens Road
Teddington
Middlesex
TW11 0LY

Leica Qwin
Leica Microsystems UK Ltd
Milton Keynes
Bedfordshire

Millipore (UK) Ltd
Walters Chromatography Division
11-15 Peterborough Road
Harrow
HA1 2YH

Mettler Toledo Ltd
64 Boston Road
Beumont Leys
Leicester
LE4 1AW

Perkin Elmer Ltd
Post Office Lane
Beaconsfield
Bucks
HP9 1QA

Phenomenex
Melville House
Queens Avenue
Hurdsfield Industrial Estate
Macclesfield, Cheshire
SK10 2BN

Promega UK Ltd
Delta House
Chilworth Science Park
Southampton
SO16 7NS

Qiagen House
Flemming Way
Crawley
West Sussex
RH10 9NQ

SGE Europe Ltd
1 Potters Lane
Kiln Farm
Milton Keynes
MK11 3LA

Sigma Aldrich Chemicals Company Ltd
Fancy Road
Poole
Dorset
VH17 7NH

Turnbull Control Systems Ltd (TCS)
Eurotherm International
Broadwater Trading Estate
Worthing, Sussex
BN14 8NW

Unicam Ltd
York Street
Cambridge
CB1 2PX

Vivascience Ltd
Customer Service UK
Epsom
Surrey

Mettler Toledo Ltd
64 Boston Road
Beumont Leys
Leicester
LE4 1AW

Perkin Elmer Ltd
Post Office Lane
Beaconsfield
Bucks
HP9 1QA

Phenomenex
Melville House
Queens Avenue
Hurdsfield Industrial Estate
Macclesfield, Cheshire
SK10 2BN

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Surrey